THE ACTION OF THE V-*ERBA* ONCOGENE WITHIN THE REGULATORY AND STRUCTURAL NETWORKS OF *XENOPUS LAEVIS* OOCYTES

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Sylvia B. Nagl

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To my parents, Kurt and Lieselotte, and in memory of my grandfather

Josef Gustav Kurt Nagl, M. D. (1918, Charles University, Prague)

The universe is infinite in all directions, not only above us in the large but also below us in the small. If we start from our human scale of existence and explore the content of the universe further and further, we will finally arrive, both in the large and in the small, at misty distances where first our senses and then even our concepts fail us.

Emil Wiechert, physicist (1896)

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Abstract

Expression in *Xenopus* oocytes revealed differential modes of regulation of the constitutive *trans*-activation domains present in the thyroid hormone receptor (TR) and its oncogenic homolog v-ErbA. The nuclear environment of oocytes induced a constitutive *trans*-activation function in TR, but not v-ErbA, at hormone-inducible thyroid hormone response elements (TREs), which was further enhanced by thyroid hormone at a subset of TREs. In contrast, DNA-protein interactions at a response element which is induced by unliganded TR in mammalian cells mediated constitutive *trans*-activation by both TR and v-ErbA. These findings indicate that the responses of the ligand-independent *trans*-activation domains of TR and v-ErbA to cell-specific and TRE-mediated induction are not equivalent. Pre-injection of nuclear protein extract from anterior pituitary cells converted v-ErbA into a strong constitutive activator at a response element from the rat growth hormone gene. Thus, the dominant negative phenotype of v-ErbA can be abolished by direct or indirect interactions with tissue-specific proteins.

The growth-promoting properties of the v-*erbA* oncogene have so far exclusively been linked to dominant repression of the anti-mitogenic roles of TR and retinoic acid receptor. Ultrastructural analysis revealed that, when expressed in *Xenopus* oocytes, v-ErbA induced early to intermediate meiotic changes occurring prior to chromosome condensation. The effects of v-ErbA on cell cycle reentry in oocytes were not mimicked by a dominant negative mutant of TR, suggesting that v-ErbA did not initiate meiosis by antagonizing endogenous TR. v-ErbA-induced meiosis occurred independently of the cAMP/MPF signal pathway, but required mRNA synthesis and translation. These findings suggest that v-ErbA mediated the release from G_2 arrest by activating expression of a cell cycle inducer(s).

I.

Introduction

1. General Introduction

(1) *Cancer as a disease of cellular signalling networks*

The question of what causes cancer is central to biology, since malignancy represents disturbances in two fundamental processes of higher organisms. These two characteristics are, firstly, the ability to proliferate or make identical copies of individual cells and, secondly, the ability to differentiate, which allows cells with a shared genetic make-up to perform functions which are extremely varied and complex. Cancer can be viewed as a disease of the signalling networks through which growth factors and other mitogenic stimuli cause cells to proliferate and differentiate. Defects leading to abnormal regulation of any of the network components, encoded by proto-oncogenes and tumour suppressor genes, may result in uncontrolled cell growth and neoplasia (Weinberg, 1989a).

An oncogene is a dominantly acting gene of cellular or viral origin that can induce one or more features of neoplastic transformation when introduced into cells, either alone or in combination with another gene. Normal cellular genes that can be converted to active oncogenes by mutation are called proto-oncogenes. Activated

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Abbreviations used in this chapter : AEV, avian erythroblastosis virus; AP-1, activator protein-1; DR4, direct repeat of TRE half sites separated by 4 nucleotides; MAPK, mitogen-activated protein kinase; MPF, maturation-promoting factor; RAR, retinoic acid receptor; T_3, 3,5,3′-L-triiodothyronine, thyroid hormone; TR, thyroid hormone receptor; TRE, thyroid hormone response element.

proto-oncogenes are thought to provide continuous proliferation signals that enhance cell growth. For example, the oncogenic protein tyrosine kinase of Rous sarcoma virus, $pp60^{v\text{-}src}$, transforms cells through unregulated phosphorylation of cellular proteins (reviewed in Hunter, 1989); while the oncogenic activity of $p21^{ras}$ proteins, carried by murine sarcoma viruses, correlates with abnormal signal transduction by these proteins, resulting from a reduction in their GTPase activity (reviewed in McCormick, 1989). Another class of oncogenes encodes factors involved in gene transcription, such as the v-*jun* and v-*fos* products of two murine retroviruses, whose cellular homologs make up the AP-1 transcription complex. In infected mice, v-Jun and v-Fos induce sarcomas by acting as continuously active transcription factors (reviewed in Eisenman, 1989; Hunter, 1989).

Tumour suppressor genes, on the other hand, are recessive genes that may counter the effects of oncogenes by either suppressing proliferation or inducing differentiation. For example, retinoblastoma, a cancer of embryonal retina cells, arises through the loss of both functional RB-1 alleles (Cavenee *et al.*, 1983; Knudson, 1971); and Wilms' tumour, a cancer of embryonal kidney stem cells, is associated with the inactivation of both copies of the WT1 tumour suppressor gene (Call *et al.*, 1990; Gessler *et al.*, 1990; reviewed in Feinberg, 1994). Tumour suppressor genes can contribute to neoplastic transformation when they become inactivated by dominant negative mutations, leading to the expression of abnormal proteins that inhibit the functions of their normal counterparts.

In this present study, novel modes of action of the v-*erbA* oncogene and of its cellular progenitor, the thyroid hormone receptor (TR), were characterized. v-*erbA*, carried by the avian erythroblastosis virus (AEV), constitutes a prototypic example of

a dominant negative oncogene, derived from a tumour suppressor gene. v-ErbA antagonizes the action of its cellular counterpart, c-*erbA*α, the chicken TRα (Damm *et al.*, 1989; Sap *et al.*, 1989; Weinberger *et al.*, 1986). TRα, a hormone-regulated transcription factor involved in the control of diverse cellular processes, has been classified as a tumour suppressor, because it helps regulate the differentiation of haematopoietic progenitor cells (Disela *et al.*, 1991; Fuerstenberg *et al.*, 1992; Gandrillon *et al.*, 1994; Rascle *et al.*, 1994; Schroeder *et al.*, 1990, 1992a, 1992b; Zenke *et al.*, 1988; 1990; reviewed in Damm, 1993). Thyroid hormone (3,5,3′-L-triiodothyronine; T_3), being a relatively small, lipophilic molecule, diffuses into cells through the plasma membrane and binds to TRs in the nucleus (reviewed in Brent *et al.*, 1991; Glass and Rosenfeld, 1991). Thus, by linking the extracellular hormonal signal directly to transcriptional processes, TR acts as an abbreviated signal transduction pathway.

(2) *Retroviral oncogenes are essential tools for contemporary oncogene research*

The RNA tumour viruses, now known as retroviruses, were among the first recognized oncogenic agents of any kind. Viruses of this type, for example the Rous sarcoma virus, were first identified as leukemogenic and sarcomagenic agents in chickens 84 years ago (Rous, 1911). In 1935, Rothe-Meyer and Engelbreth-Holme reported the identification of another infectious agent capable of inducing erythroleukemia in birds (reviewed in Privalsky, 1992). Several isolates of the "avian erythroblastosis virus" were obtained, and ultimately a stable strain, designated AEV-ES4/R, was recovered and shown to be a retrovirus. An intensive investigation of the oncogenic mechanisms employed by these viruses, however, began only in

contemporary times, made possible by the development of assays for transformation of cultured cells and biochemical methods for analysis of viral genomes and proteins. Consequently, four decades elapsed before the study of AEV-ES4/R was resumed (Graf *et al.*, 1976; Graf and Beug, 1978), with a subsequent focus on the cooperative action of the two AEV-associated oncogenes, v-*erbA* and v-*erbB* (Graf and Beug, 1983; Frykberg *et al.*, 1983; Vennström and Bishop, 1982).

Highly oncogenic retroviruses, including AEV, are the most rapid and efficient oncogenic agents known (reviewed in Teich *et al.*, 1985). Viral oncogenes therefore provide a good experimental model system to study oncogenesis. Furthermore, the widespread presence of activated oncogenes in human tumours suggests that there are common mechanisms governing transformation by oncogenic retroviruses and oncogenesis by other carcinogenic agents (reviewed in Varmus, 1989). The rates of base substitutions for retroviral genes is roughly 10^6 times higher than that for their cellular counterparts (Gojobori and Yokoyama, 1985). The high rate for the retroviral genes can be attributed to a high mutation rate caused in the process of reverse transcription (Temin, 1989), and potentially allows retroviruses to undergo multiple mutations and recombinations before they are subject to selection. These observations have given rise to the hypothesis that the evolution of oncogenic retroviruses is a mutation-driven process, whose stages are analogous to the spontaneous mutations leading to the activation of cellular proto-oncogenes (Temin, 1988; reviewed in Weinberg, 1989b). In nonretroviral cancers, genetic instability and the concomitant accumulation of mutations results in tumour cell heterogeneity. This process provides malignant tumours with great biological diversity, which enables them to succeed in a competitive environment. Consequently, cancer cells not only

develop aggressive proliferation and metastasizing potential, but also resistance to natural and synthetic drugs (reviewed in Pienta *et al.*, 1989). The explanatory power of the "oncogene hypothesis" has provided a strong impetus to efforts aimed at understanding the precise role of mutations found in retroviral and cellular oncogenes.

(3) *Contributions of v/c-erbA research to an understanding of human cancer*

It was recently reported that male mice transgenic for v-*erbA* develop hepatocellular carcinoma, demonstrating that v-*erbA* can promote neoplasia in mammals (Barlow *et al.*, 1994). Based on the fact that v-ErbA contributes to oncogenic transformation by dominantly repressing TR and the closely related retinoic acid receptor (RAR) (Schroeder *et al.*, 1992a, 1992b), dominant negative forms of TR and RAR could reasonably be expected to be involved in human cancers. Dominant negative forms of TR, with several genetic lesions resembling those present in v-ErbA, have been identified in the rare syndrome of generalized thyroid hormone resistance (GTHR) (reviewed in Privalsky, 1992). In TRs from individuals suffering from GTHR, point mutations in the ligand binding domain result in ligand binding-deficient receptors that act as dominant negative inhibitors when coexpressed with the wild-type TR. Elevated rates of cancer development have not been observed in affected individuals, suggesting that the expression of at least these particular dominant negative TR alleles does not result in cancer in humans. However, the possibility that a link between other dominant negative forms of TR and human cancer might be found in the future cannot yet be excluded. Interestingly, a dominant negative RAR has been implicated in promyelocytic

leukemia (PML) in humans (reviewed in Damm, 1993; Privalsky, 1992). The unliganded PML-RARα fusion product, consisting of a truncated RARα gene translocated to the PML locus on chromosome 15, is thought to act through a dominant negative mechanism to interfere with promyelocytic differentiation. This indicates that inhibition of cellular differentiation by dominant negative nuclear receptors of cellular origin does indeed occur in human cancers.

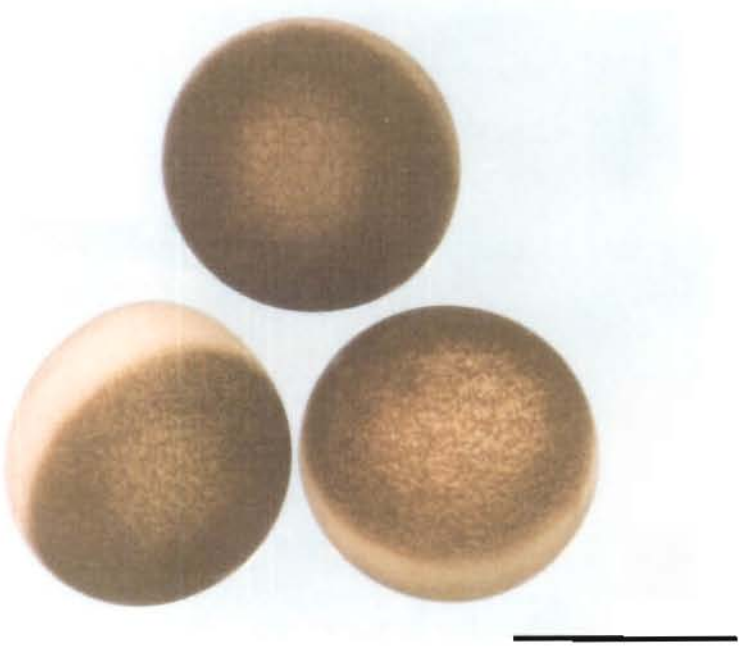
An understanding of the processes which help to prevent cancer is one of the primary aims of cancer research. In this regard, perhaps the most important outcome of studies of v/c-*erbA* to date has been the characterization of c-*erbA* as a tumour suppressor gene (reviewed in Damm, 1993). Not only does TR/c-ErbA mediate differentiation of erythroid progenitor cells, but it also is involved in: (i) repression of mitogenic signalling components, such as repression of the AP-1 transcription factor complex in chicken embryo fibroblasts (Desbois et al., 1991a, 1991b; Zhang *et al.*, 1991), and inhibition of platelet-derived growth factor B/c-*sis* gene expression in glial cells (Iglesias *et al.*, 1995); (ii) inhibition of proliferation (Gandrillon *et al.*, 1994); and (iii) induction of programmed cell death (apoptosis) (Gandrillon *et al.*, 1994). Finally, the study of v-ErbA has been invaluable in the molecular dissection of functional domains of nuclear receptors, and has greatly contributed to our understanding of the action of dominant negative oncogenes. These aspects of v-ErbA are discussed in detail in Chapters II and III.

(4) *The Xenopus laevis oocyte is a model system for studies of transcriptional regulation and oncogene action*

Oocytes of *Xenopus laevis*, the African clawed frog, can be used as an exceptionally efficient assay system to characterize the function of both *cis*- and *trans*-acting factors in gene expression (Fig. 1) (Gurdon and Melton, 1981; Heikkila, 1990; Wang *et al.*, 1991). For example, *Xenopus* oocytes have recently been employed in studies of transcriptional regulation by oestrogen receptors (Theulaz *et al.*, 1988; McKenzie *et al.*, 1990; Watson, 1991), and by a recombinant *trans*-activator protein, which consisted of the potent activation domain of the herpes simplex virus virion protein VP16 and the DNA binding domain of the yeast activator GAL4 (GAL4-VP16) (Xu *et al.*, 1993). Unlike expression studies in transfected cultured cells, oocyte microinjection offers the possibility to directly introduce cell components, such as nuclear proteins, as well as gene templates, and thereby allows the *in vivo* reconstitution of cell-specific nuclear environments. This advantage of the *Xenopus* oocyte expression system is illustrated by studies of ß-globin promoter activation in the presence of nuclear extracts from erythroid tissues (Rungger *et al.*, 1990); silencing and activation of the mouse interleukin-2 gene promoter by protein extracts from resting and mitogen-induced primary T-lymphocytes (Mouzaki *et al.*, 1991); and stimulation of sea urchin early and late H2B histone gene expression by a gastrula nuclear extract (Maxson *et al.*, 1988).

Oncogene involvement in the signal transduction events mediating control of the cell cycle can be studied by maturation assays carried out in *Xenopus* oocytes (reviewed in Smith, 1989). For example, the oncogenic $p21^{ras}$ product (Birchmeier *et al.*, 1985; Pomerance *et al.*, 1992), the v-*mos* protein (Freeman *et al.*, 1990), the

Figure 1. Stage V (Dumont, 1972) *Xenopus laevis* oocytes. The dark pigmented animal pole and the unpigmented vegetal hemisphere are shown. Bar: 1 mm.



$pp60^{v\text{-}src}$ product (Spivack *et al.*, 1984), and the oncogenic form of *neu* tyrosine kinase (Narasimhan *et al.*, 1992) have been studied in oocytes. Unlike most vertebrate somatic cells, which are arrested in G_0, *Xenopus* oocytes are arrested in prophase at the G_2/M border of meiosis. Progesterone stimulation of oocytes leads to the activation of pre-existing mitogen-activated protein kinase (MAPK) and a cyclin B/CDC2 complex, known as maturation-promoting factor (MPF), and subsequent resumption of the cell cycle (reviewed in Kosako *et al.*, 1994a; Maller, 1990; Smith, 1989). Since expression of oncogenes, or microinjection of oncogenic proteins, can also lead to cell cycle reentry, oocytes offer a useful system in which the pathways mediating oncogene action can be analyzed.

2. Nature and Scope of this Investigation

(1) *Aim 1: How do the trans-activation and repression domains of TR and v-ErbA function in Xenopus oocytes?*

A central issue arising from the discovery of retroviral oncogenes is how a viral oncogene differs functionally from its normal progenitor. To address this question, the potential for differential cell-specific modes of action distinguishing TR from its retroviral homolog v-ErbA was tested in *Xenopus* oocytes. For the characterization of TR and v-ErbA function, regulatory networks, involving TRs, mammalian and avian thyroid hormone response elements (TREs), and cell-specific nuclear protein extracts, were reconstituted in oocytes. The findings of this study are described and discussed in Chapter II.

(2) ***Aim 2: Does v-ErbA initiate cell cycle reentry in Xenopus oocytes?***

The growth-promoting properties of the v-*erbA* oncogene have so far exclusively been linked to dominant repression of the anti-mitogenic roles of TR and RAR (Desbois *et al.*, 1991a, 1991b; Gandrillon *et al.*, 1994; Schroeder *et al.*, 1992a, 1992b; Sharif and Privalsky, 1991; Zhang *et al.*, 1991). Chapter III describes the results of meiotic induction assays and ultrastructural studies in *Xenopus* oocytes, which showed that the v-*erbA* product induces early to intermediate meiotic events by activating *de novo* gene expression. The findings of this study suggest that v-ErbA does not initiate cell cycle reentry by antagonizing endogenous TR in oocytes.

II.

Constitutive *Trans*-activation by the Thyroid Hormone Receptor and a Novel Pattern of Activity of its Oncogenic Homolog v-ErbA in *Xenopus* Oocytes

1. Introduction

This chapter describes how transcriptional control by the α and ß isoforms of thyroid hormone receptor (TR) is modulated by (i) the nuclear environment of *Xenopus* oocytes, (ii) the structure of the DNA control elements, termed thyroid hormone response elements (TREs), at which TRs are bound, and (iii) heterodimerization with the retinoid X receptor (RXR). The oncogenic counterpart of TR, v-ErbA, was tested for its ability to dominantly repress TR at a range of TREs in the context of *Xenopus* oocytes. v-ErbA was shown to be converted to a transcriptional activator by binding to certain TREs and by the presence of injected tissue-specific factors from anterior pituitary cells. In the introduction, the structural and functional differences between TRα, TRß, and v-ErbA, and the mechanisms of transcriptional control by these nuclear receptors are reviewed.

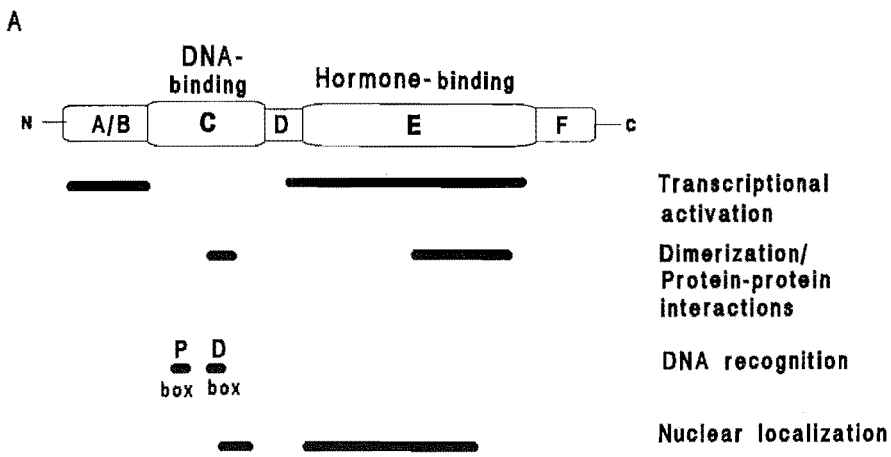
Abbreviations used in this chapter : ELISA, enzyme linked immunosorbant assay; lysTRE, TRE from the chicken lysozyme gene; malTRE, TRE from the rat malic enzyme gene; rGH, rat growth hormone; rGH_3TRE, TRE from the third intron of the rat growth hormone gene; RXR, retinoid X receptor; T_3, 3,5,3′-L-triiodothyronine, thyroid hormone; TR, thyroid hormone receptor; TRE, thyroid hormone response element; TREp, palindromic TRE.

(1) *Evolutionary relationships and diversity of thyroid hormone receptor genes*

TRs are members of the steroid/thyroid hormone receptor gene family of sequence-specific transcription factors, inducible by their cognate ligands (Detera-Wadleigh and Fanning, 1994; Laudet *et al.*, 1992). In addition to receptors for thyroid hormone (T_3), members of this family include the receptors which mediate the effects of glucocorticoids, androgens, oestrogens, progestins, mineralocorticoids, 1,25-dihydroxyvitamin D_3, all-*trans* retinoic acid, and 9-*cis* retinoic acid, as well as a number of related orphan receptors without known ligands (Detera-Wadleigh and Fanning, 1994; Laudet *et al.*, 1992; reviewed in Beato, 1989; Evans, 1988). Nuclear hormone receptors are key regulators of metabolism, reproduction, development and tissue-specific differentiation (reviewed in Beato, 1989; Chatterjee and Tata, 1992; Evans, 1988). Recently, a nuclear receptor, named farnesoid X-activated receptor, was characterized which is activated by the intracellular metabolite farnesol, a component of the mevalonate pathway in mammalian cells (Forman *et al.*, 1995a). Interestingly, the isolation of this receptor suggests the existence of a potentially novel metabolite-controlled intracellular signalling system in vertebrates.

TRs, like other transcription factors, are composed of modular functional domains whose action is dependent on cellular context (domains A to F; Fig. 2A) (reviewed in Glass and Rosenfeld, 1991; Green and Chambon, 1986). TRs are encoded by two distinct but closely related genes, *c-erbA*α and c-*erbA*ß, which map to chromosomes 17 and 3 respectively in humans (reviewed in Brent *et al.*, 1991). Both genes express several alternatively spliced isoforms. The TRα genes in rats and humans express the T_3-binding isoform TRα1 along with c-ErbAα2, which does not

Figure 2. Modular composition and amino acid sequence similarity of TR isoforms and v-ErbA. (*A*) Schematic of a generalized TR represented as a rectangle; indicated within are the A/B, C, D, E and F subregions. Below are shown the various receptor domains thought to be involved in transcriptional activation, dimerization or interaction with other proteins, DNA recognition and binding (including the *P*- and *D*-box elements), and the regions thought to mediate nuclear localization (adapted from Privalsky, 1992). (*B*) Comparison of primary structures of human TRß1 (hTR beta1), rat TRα1 (rTR alpha1) and v-ErbA, the proteins used in this study. v-*erbA* is expressed as a fusion protein made up of the retroviral *gag* protein and the v-*erbA* product. Numbers within regions corresponding to the C and E domains represent percent amino acid identity with hTRß1 (adapted from Glass and Rosenfeld, 1991). Little or no significant homology is observed when comparing other regions of the receptors.


A
DNA-
binding
Hormone-binding
N
A/B
C
D
E
F
C
Transcriptional
activation
Dimerization/
Protein-protein
interactions
P
box
D
box
DNA recognition
Nuclear localization
B
A/B
C
D
E
100
100
hTR beta1
90
87
rTR alpha1
gag
88
86
v-ErbA

bind T_3 because of alternative splicing at the C terminus (reviewed in Brent *et al.*, 1991; Glass and Rosenfeld, 1991). The closely related chicken α gene expresses only chicken TRα which is over 90% identical at the amino acid level to rat TRα1 (rTRα1) and human TRα1 (hTRα1) (Brent *et al.*, 1991). The TRß gene expresses TRß1 and TRß2 that differ only in their N-terminal A/B regions, which are distinct from the A/B region of TRα1 (Brent *et al.*, 1991; Glass and Rosenfeld, 1991). rTRα1 and hTRß1, employed in this study, are over 85% identical at the amino acid level in their DNA binding and ligand binding domains (Fig. 2B).

(2) *The role of thyroid hormone receptors in the regulation of eukaryotic gene transcription by RNA polymerase II*

Cells regulate the transcription of virtually all their genes; for example, in order to establish a differentiated phenotype during development, or in response to extracellular signals, such as T_3. Transcription of eukaryotic protein-encoding genes by RNA polymerase II involves the assembly of multiprotein complexes on distal control DNA elements, called enhancers, and proximal promoters (reviewed in Maldonado and Reinberg, 1995). The highly regulated nature of transcription complex assembly provides a finely tuned mechanism for controlling gene expression. Transcription is mediated by two distinct classes of transcription factors. The first class comprises general transcription factors which are necessary for accurate initiation of transcription. These factors include TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIG/J, and TFIIH (reviewed in Maldonado and Reinberg, 1995; Buratowski and Sharp, 1992). TFIID is a multiprotein complex consisting of the TATA box-binding protein (TBP) complexed with a number of TBP-associated

factors (TAFs) (Maldonado and Reinberg, 1995). The binding of TFIID is thought to be the first step in transcriptional initiation, which is followed by the stepwise recruitment of the additional transcription factors and RNA polymerase II, and ultimately results in the initiation of transcription (Pugh and Tjian, 1992).

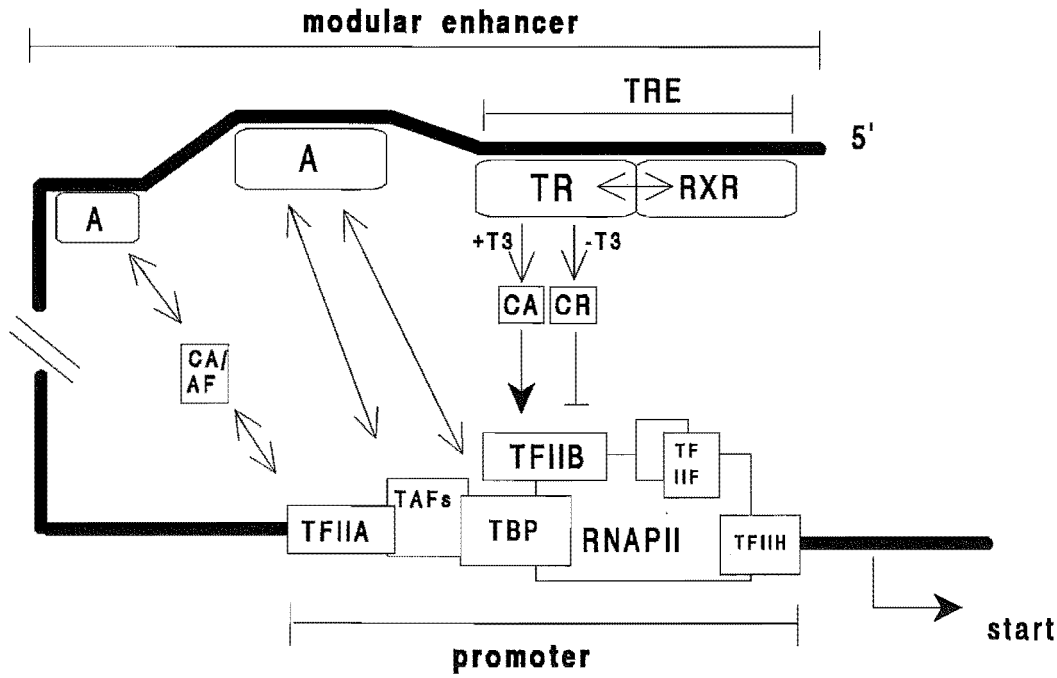
The magnitude of transcriptional activation is greatly affected by the second class of sequence-specific transcription factors, transcriptional activators and repressors, which act to either promote or inhibit the formation of an active transcriptional initiation complex (reviewed in Johnson, 1995; Ptashne, 1988; Ptashne and Gann, 1990; Tjian and Maniatis, 1994). Transcriptional activators bind to enhancers, and thereby select genes to be activated, and orchestrate the assembly of the general transcription "machinery" at the start site of mRNA synthesis by RNA polymerase II (Fig. 3) (Maldonado and Reinberg, 1995; Tjian and Maniatis, 1994). Enhancer elements, which include DNA binding sites for TR, can be located at great distances from the start site of transcription, either upstream, downstream or within a transcribed gene (Banerji *et al.*, 1981; Moreau *et al.*, 1981). Transcription factors bound to enhancers activate transcription by forming direct protein-protein contacts with proximal promoter factors through DNA looping (Dunn *et al.*, 1984; reviewed in Serfling *et al.*, 1985).

Recent *in vitro* transcription studies suggest that the binding of TFIIB may be the rate-limiting step for the initiation of transcription, and that several transcriptional activators act to recruit or stabilize the interaction of TFIIB within the initiation complex (Hori *et al.*, 1995; Maldonado and Reinberg, 1995; Ptashne and Gann, 1990). Protein-protein interactions with the general transcription factor TFIIB have been implicated both in transcriptional repression by unliganded TR and

transcriptional activation in response to ligand (Fig. 3) (Fondell *et al.*, 1993; Baniahmad *et al.*, 1993). A short sequence in the N-terminal A/B region of DNA-bound TRα is required for efficient binding to TFIIB and acts to recruit and/or stabilize the binding of TFIIB to the transcription complex (Hadzic *et al.*, 1995). For transcriptional repression, however, inhibitory interactions with TFIIB are not sufficient; the recently identified corepressor N-CoR is also required (Baniahmad *et al.*, 1992; Hörlein *et al.*, 1995).

A central question in understanding transcriptional regulation is how a relatively small number of different transcription factors can be used to achieve the high level of specificity required to regulate the complex patterns of gene expression in higher eukaryotes. At least part of the answer is that transcription factors and enhancers are composed of modular components (reviewed in Beato, 1989; Dynan, 1989; Chatterjee and Tata, 1992; Tjian and Maniatis, 1994; Gronemeyer and Moras, 1995). As exemplified by TR (Fig. 2A), a typical transcription factor contains a specific DNA-binding domain, a multimerization domain that allows the formation of homodimers or heterodimers, and a transcriptional *trans*-activation domain. Similarly, enhancers contain distinct sets of transcription factor-binding sites, and variations in the arrangement of the binding sites provide the potential to create unique DNA-protein complexes by forming heterodimers within and among families of transcription factors (reviewed in Karin et *al.*, 1993; Mangelsdorf and Evans, 1992). Synergistic interactions between the proteins within the complex result in specificity, a potential for multiple regulatory controls, and a high level of transcription. Importantly, cell-specific cofactors modulate the effects of transcriptional activators and repressors in a highly tissue-specific manner.

Figure 3. Schematic of a multistep model of *trans*-activation by TR. The multistep model of *trans*-activation is derived mainly from *in vitro* studies of mammalian systems (adapted from Maldonado and Reinberg, 1995). Multiple cooperative interactions (symbolized by arrows) between TR, RXR, other activators (A), coactivators (CA), corepressors (CR), accessory factors (AF), and general transcription factors (TFIIA-H, TBP, TAFs), mediate activation of transcription by RNA polymerase II (RNAPII) at the initiation start site (start) of TR-regulated genes. The multiprotein complex made up of TATA-binding protein (TBP) and TBP-associated factors (TAFs) constitutes the general transcription factor TFIID. In mammalian cells, TR, associated with CR, represses transcription by inhibitory interactions with TFIIB in the absence of T_3 (-T3). T_3 induces both the dissociation of CR and the recruitment of CA (+T3). As a result, the TR/CA complex activates transcription by enhancing binding of TFIIB to the general transcription complex (refer to text for further details).


modular enhancer
TRE
5'
A
A
TR
RXR
+T3
-T3
CA
CR
CA/
AF
TFIIB
TF
IIF
TAFs
TFIIA
TBP
RNAPII
TFIIH
start
promoter

These mechanisms are well illustrated by the multi-level control of the rat growth hormone (rGH) gene, which offers an interesting system for studying the interactions between factors that mediate cell-type specificity and the factors that mediate hormone responsiveness. The promoter of the rGH gene, which is exclusively expressed in the somatotrophic cells of the anterior pituitary gland (reviewed in Frawley, 1989), contains DNA binding sites for TR (Ye *et al.*, 1988; Flug *et al.*, 1987; Norman *et al.*, 1989; Brent *et al.*, 1989) and for the general transcription factors Sp1 (Schaufele *et al.*, 1990a) and GHF-3 (Schaufele *et al.*, 1990b), as well as two sites for the pituitary-specific factor Pit-1/GHF-1, belonging to the homeobox POU domain-containing family of transcription factors (Bodner *et al.*, 1988; Lefevre *et al.*, 1987; Schaufele *et al.*, 1990c; West *et al.*, 1987). An additional binding site for TR, which was employed in this present study, is located in the third intron of the rGH gene (Sap *et al.*, 1990). TR and Pit-1 act synergistically in the activation of the rGH promoter (Schaufele *et al.*, 1992; Ye *et al.*, 1988), which helps to explain how the widely distributed TR can activate the expression of certain genes only in selected cell types.

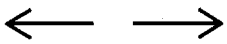
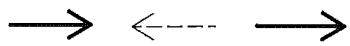
(3) *Specific recognition of thyroid hormone response elements by thyroid hormone receptors*

The DNA binding domain of TR possesses two zinc finger motifs (Freedman *et al.*, 1988; Luisi *et al.*, 1991). Adjacent to each zinc finger is an amphipathic α-helix, involved in the folding of the zinc finger domains into a compact globular structure (Hard *et al.*, 1990a and 1990b; Luisi *et al.*, 1991; Remerowski *et al.*, 1991; Schwabe *et al.*, 1990). The *P*-box of the first α-helix, mediating TRE recognition, contains

three discriminating amino acids thought to interact directly with specific bases of the consensus motifs within TREs (Danielsen *et al.*, 1989; Luisi *et al.*, 1991; Mader *et al.*, 1989; Umesono *et al.*, 1989). Further dissection of the *P*-box revealed that the amino acids act independently at the level of DNA binding (Nelson *et al.*, 1993). The *D*-box, adjacent to the second α-helix, provides phosphate contacts to the DNA backbone and participates in receptor-receptor dimerization (Danielsen *et al.*, 1989; Lee *et al.*, 1993; Luisi *et al.*, 1991; Umesono and Evans, 1989; Umesono *et al.*, 1991; Zechel *et al.*, 1994a, 1994b).

TRs control expression of T_3-responsive genes at *cis*-acting TREs (reviewed in Chatterjee and Tata, 1992). TREs are made up of six base pair consensus sequences, termed half sites, consisting of the canonical 5'-PuGGTCA recognition motif. These half-sites can be oriented as imperfect inverted, direct or divergent repeats, separated by different sized nucleotide gaps (reviewed in Gronemeyer and Moras, 1995; Privalsky, 1992). Naturally occurring promoters often show mixtures of half sites in differing orientations (Chatterjee and Tata, 1992). Specific recognition of a TRE by the receptor appears to require the appropriate orientation and spacing of the half-sites, as well as the correct sequence within each half-site (Baniahmad *et al.*, 1990; Desai-Yajnik and Samuels, 1993; Forman *et al.*, 1992; Glass *et al.*, 1988; Lazar *et al.*, 1991; Näär *et al.*, 1991; Umesono *et al.*, 1991). TR homodimers specifically recognize a palindromic arrangement of half-site motifs with no spacer nucleotide (reviewed in Gronemeyer and Moras, 1995), as exemplified by a synthetic response element, termed TREp (Damm *et al.*, 1989) used in this study (Fig. 4). This requirement for a distinct number of spacer nucleotides results from the characteristics of the *D*-box within the DNA binding domain, which represents a

Figure 4. Thyroid hormone response elements employed in this study. Nucleotide sequences of the TRE from the rat malic enzyme gene (malTRE) (Petty *et al.*, 1990), the TRE from the chicken lysozyme gene (Baniahmad *et al.*, 1990), the TRE from the third intron of the rat growth hormone gene (rGH_3TRE) (Sap *et al.*, 1990), a synthetic palindromic TRE (TREp) (Damm *et al.*, 1989), and the clone 122 TREs (Bigler and Eisenman, 1994) are shown. The orientation of individual half sites is indicated by arrows. Intervening nucleotides between half-sites are indicated by numbers. A putative half site within rGH_3TRE is depicted by a broken arrow.

TRE:	*type:*	*gene:*
 GGGTTA 4 AGGACA	direct repeat DR4	*rat* malic enzyme
 CCCCAG 2 TGAGGT	inverted palindrome	*chicken* lysozyme
 AGGTAA 6 AGTCCC 6 AGGTCA	tripartite	*rat* growth hormone (third intron)
 AGGTCA 0 TGACCT	palindrome	synthetic construct
TRE1: TAAGCCCCCAGCCCCCCGACAT CCAGGACGCCCCAAA TRE2:  ATACCTTATTACCTCATCATGTGAAATAG TRE3:  TCCGAGTGGACTCGGCTCGGTCATTGGGTG	complex	"clone 122" gene unknown

weak DNA-induced dimerization interface (Luisi *et al.*, 1991; Schwabe *et al.*, 1990; Umesono and Evans, 1989). TRs can bind TREs as monomers and homodimers (Forman *et al.*, 1992; Ikeda *et al.*, 1994; Lazar *et al.*, 1991; Katz and Koenig, 1993). However, heterodimerization with mammalian auxiliary factors, most notably the nuclear receptors for 9-*cis* retinoic acid (RXRs), results in increased affinity for the response element *in vitro* and enhanced activation of gene expression *in vivo* (Bugge *et al.*, 1992; Burnside *et al.*, 1990; Heyman *et al.*, 1992; Kliewer *et al.*, 1992; Murray and Towle, 1989; Yu *et al.*, 1991; Zhang *et al.*, 1992; for a review, see Mangelsdorf and Evans, 1992).

Many naturally occurring TREs are composed of two directly repeated half-sites separated by four nucleotides (DR4), such as the TRE from the rat malic enzyme gene (malTRE) (Petty *et al.*, 1990) employed in this study (Fig. 4). Heterodimerization of TR with RXR at DR4, which is due to a strong interaction between the dimerization surfaces located in the ligand binding domains and weaker interaction between the dimerization surfaces in the *D*-boxes of TR and RXR, results in cooperative DNA binding and specific recognition of DR4 TREs (Kliewer *et al.*, 1992; Lee *et al.*, 1993; Leid *et al.*, 1992a, 1992b; Mader *et al.*, 1993; Marks *et al.*, 1992; Perlmann *et al.*, 1993; Yu *et al.*, 1991; Zechel *et al.*, 1994a, 1994b; Zhang *et al.*, 1992). Recently, the understanding of the stereochemical principles underlying specific recognition of DR4 TREs by TR-RXR heterodimers was greatly enhanced by the crystallographic characterization of the heterodimer bound to a DR4 TRE (Rastinejad *et al.*, 1995).

In addition to palindromic and DR4 TREs, inverted palindromes which are bound by homodimers and heterodimers (Gronemeyer and Moras, 1995), like the TRE from

the chicken lysozyme gene (lysTRE) (Baniahmad *et al.*, 1990) used in this study (Fig. 4), are found in naturally occurring enhancer elements. Finally, complex TREs, consisting of more than two half-sites arranged in differing orientations, have also been described. Two complex TREs, the TRE from the third intron of the rat growth hormone gene (rGH_3TRE) (Sap *et al.*, 1990), and the clone 122 TREs of an as yet unidentified T_3-responsive gene (Bigler and Eisenman, 1994), were employed in this investigation (Fig. 4). At present, it remains unknown whether TR binds to complex TREs in monomeric, dimeric, or multimeric form.

(4) *Constitutive and ligand-induced transcriptional regulation by thyroid hormone receptors*

Induction of gene transcription by TR is achieved through autonomous *trans*-activation domains (reviewed in Gronemeyer, 1992): a constitutive *trans*-activation domain located in the N-terminal region of the receptor (Hadzic *et al.*, 1995; Hollenberg *et al.*, 1995) and, in the C-terminus, three ligand-inducible *trans*-activation domains, which are all required for maximum transcriptional activation (Baniahmad *et al.*, 1995). Although transcriptional activation by TR is primarily ligand-dependent in mammalian cells, ligand-independent activation has also been observed (Bigler and Eisenman, 1994; Forman *et al.*, 1988; Forman and Samuels, 1990; Saatcioglu *et al.*, 1993a). In the yeast, *Saccharomyces cerevisiae*, TR functions as a ligand-independent activator with enhanced expression in the presence of T_3 (Privalsky *et al.*, 1990). In mammalian cells, unliganded TRs, thought to be constitutively bound to hormone response elements, can suppress the basal activity of promoters containing TREs (Baniahmad *et al.*, 1990, 1992; Damm *et al.*, 1989; Fondell *et al.*,

1993; Sap *et al.*, 1989; Zenke *et al.*, 1990). The repressor function has been mapped to the hinge domain of TR, and was shown to be separable from its activator function (Damm and Evans, 1993). A C-terminal silencer domain has also been characterized (Baniahmad *et al.*, 1992, 1995). At present, it is not well understood how the *trans*-activation and repression domains are regulated in the context of the full-length receptor.

At positively regulated TREs, addition of T_3 results in the activation of transcription, suggesting that the hormone triggers a conformational change in TR that induces the activator function (Allan *et al.*, 1992; Bhat *et al.*, 1993; Leng *et al.*, 1993; Toney *et al.*, 1993). This conformational switch causes both the dissociation of corepressor proteins as well as the recruitment of coactivator(s) (Chen and Evans, 1995; Hörlein *et al.*, 1995; Kurokawa *et al.*, 1995; Lee et al., 1995a, 1995b). At negatively regulated TREs, TRs act as transcriptional repressors when bound to T_3 (reviewed in Chatterjee and Tata, 1992; Glass and Rosenfeld, 1991). In addition, two novel TREs were recently identified. Both the clone 122 TREs (Bigler and Eisenman, 1994) and a TRE in the Rous sarcoma virus long terminal repeat (LTR) (Saatcioglu *et al.*, 1993a), mediate strong activation by unliganded TR, while addition of T_3 reverses this response.

(5) *v-ErbA: Origins and mode of action*

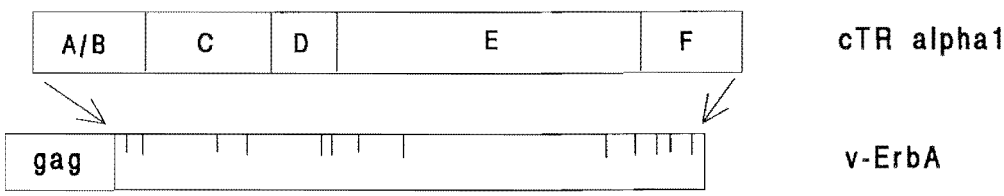
The v-*erbA* gene of the avian erythroblastosis virus (AEV) constitutes a virally transduced, highly mutated form of the chicken TRα gene, and is expressed as a fusion product with *gag*, the retroviral structural protein gene (for a review, see Privalsky, 1992). AEV belongs to a small group of avian replication-defective

leukemia viruses and carries two distinct oncogenes, *v-erbA* and *v-erbB*, which together induce erythroblastosis in young chicks (Graf *et al.*, 1976; Graf and Beug, 1983). This collaborative action of v-*erbA* and v-*erbB* is discussed in detail in Chapter III.

The cooperative mode of v-*erbA* function reinforces the notion that the development of a transformed phenotype is most readily achieved through the actions of at least two oncogenes, the hypothesis known as "the multistep causation of carcinogenesis" (reviewed in Weinberg, 1989b). Since the v-*erbA* oncogene is not itself capable of overt transformation (Gandrillon *et al.*, 1987), but cooperates with a wide variety of other oncogenes to enhance transformation (Kahn *et al.*, 1986), it provokes two intriguing questions: What is its cooperative role, and how does it exert its subtle effects on growth and differentiation?

To date, the growth-inducing properties of v-*erbA* have been shown to involve interference with the function of nuclear hormone receptors, belonging to signalling pathways distinct from those induced by the mitogenic kinases. v-ErbA acts as a dominant repressor in mammalian and avian cells, blocking activation of gene expression by two differentiation-inducing factors, liganded TR and retinoic acid receptor (RAR) (Damm *et al.*, 1989; Sap *et al.*, 1989; Sharif and Privalsky, 1991). The v-*erbA* protein has sustained small N- and C-terminal deletions and 13 internal amino acid changes relative to the TR progenitor (Fig. 5) (for a review, see Privalsky, 1992). Mutations within the C-terminus of the v-*erbA* protein appear primarily responsible for its conversion into a constitutive transcriptional repressor, as these mutations result in loss of the ability to bind thyroid hormone at high affinity (Damm *et al.*, 1989; Sap *et al.*, 1989; Zenke *et al.*, 1990; for a review, see Privalsky,

Figure 5. Comparison of the chicken TRα1 (cTR alpha1) and v-ErbA. The cTRα1 protein is displayed schematically on top, and the v-*erbA* protein is presented for comparison below. Arrows indicate the N- and C-terminal deletions relative to cTRα1, and the dashes indicate the locations of the 13 internal amino acid differences between cTRα1 and v-ErbA. In addition, the N-terminal fusion to retroviral *gag* sequences in v-ErbA is shown (adapted from Privalsky, 1992).


A/B
C
D
E
F
cTR alpha1
gag
v-ErbA

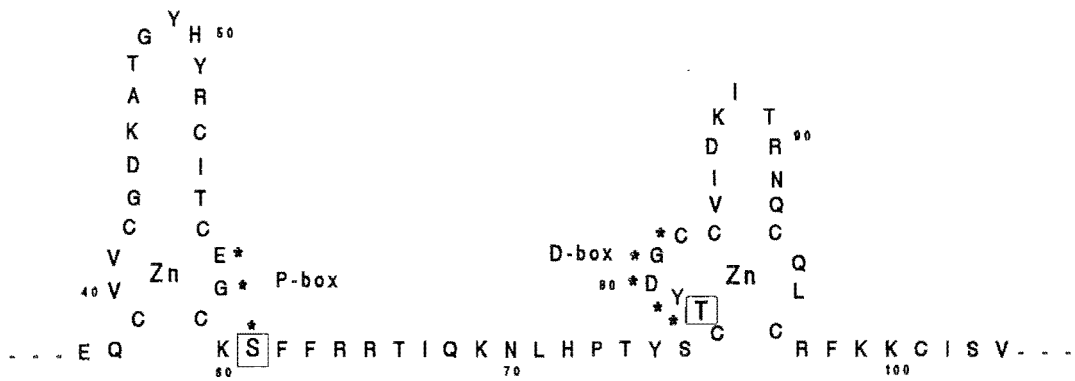
1992). v-ErbA has retained some binding ability, since, surprisingly, v-ErbA acts as a hormone-regulated activator in *S. cerevisiae* (Privalsky *et al.*, 1990; Sharif and Privalsky, 1991). The ability of v-ErbA to respond to hormone in yeast suggests that its hormone binding ability can be influenced by the environment. Consequently, cellular factors modulating hormone-receptor interactions may be important in the oncogenicity of v-*erbA*.

Several mechanisms have been suggested to account for transcriptional repression by v-ErbA, such as direct promoter silencing (Baniahmad *et al.*, 1990, 1992), competition with TR and RAR for DNA binding sites (Damm *et al.*, 1989; Selmi and Samuels, 1991), or formation of inactive heterodimers between v-ErbA and TR or RAR (Desbois *et al.*, 1991a; Schroeder *et al.*, 1992a, 1992b; for reviews, see Yen and Chin, 1994; Herskowitz, 1987). Of these mechanisms, direct promoter silencing is the least understood (reviewed in Levine and Manley, 1989; Renkawitz, 1990). That v-ErbA and unliganded TR are able to repress transcription through direct promoter silencing is shown by the fact that repression is effective on a minimal promoter (Baniahmad *et al.*, 1990, Fondell *et al.*, 1993). Moreover, both factors inhibit transcription independently of the position and orientation of the TRE to which they are bound (Baniahmad *et al.*, 1990). The following observations suggest that competition for DNA binding is critical for dominant negative activity. Firstly, mutation of the DNA binding domain of v-ErbA abrogates its dominant negative activity (Damm *et al.*, 1989). Secondly, v-ErbA homodimers, v-ErbA/RXR heterodimers and v-ErbA/TR heterodimers can form on TREs (Hermann *et al.*, 1993; Yen *et al.*, 1994). It has been proposed that one or all of these complexes may compete with normal TR complexes for binding at TREs and thereby block T_3-

mediated gene activation (Yen and Chin, 1994). Finally, these different mechanisms do not have to be mutually exclusive. For instance, inactive v-ErbA/TR dimers might form on a TRE and silence the promoter by blocking the assembly of a functional pre-initiation complex (Baniahmad *et al.*, 1990, 1992; Fondell *et al.*, 1993; Yen *et al.*, 1994; Yen and Chin, 1994). By default, they would also prevent TR complexes from binding to that TRE (Yen and Chin, 1994).

In addition to mutations in the hormone binding domain (Fig. 5), the v-*erbA* protein has acquired a substitution of one of the three discriminating amino acids within the *P*-box, and a second mutation in the *D*-box of the DNA binding domain (Fig. 6) (Bonde *et al.*, 1991; Sap *et al.*, 1986; for a review, see Privalsky, 1992). While the ability of the DNA binding domain of v-ErbA to bind to certain TRE sequences has been conserved (Bonde *et al.*, 1991; Damm *et al.*, 1989; Sap *et al.*, 1989; Verneuil and Metzger, 1990), the two zinc finger domain mutations, and mutations in an N-terminal region outside of the DNA binding domain, alter the range of DNA target sequences the v-*erbA* protein can bind to relative to TR (Chen *et al.*, 1993; Sharif and Privalsky, 1991; Smit-McBride and Privalsky, 1993; Subauste and Koenig, 1995; Zenke *et al.*, 1990). To summarize, multiple mutations and two deletions confer an oncogenic phenotype on the v-*erbA* product. v-ErbA has evolved into a dominant repressor of TR, its cellular progenitor, and also exhibits an altered range of DNA binding ability relative to TR.

Figure 6. Schematic representation of the DNA binding domain of the v-*erbA* protein. The two zinc finger modules are shown, using the standard one letter amino acid code. The relationship of the conserved cysteines (C) to the coordinated zinc atoms (Zn), involved in stabilization and folding of the two zinc finger modules, is emphasized. The amino acids comprising the *P*- and *D*-boxes are indicated by asterisks. The two amino acid substitutions sustained by v-ErbA relative to cTRα1 are boxed (adapted from Privalsky, 1992).


Y
G
H
50
T
Y
A
R
K
C
D
I
G
T
C
C
V
E
Zn
40 V
G
P-box
C
C
K
S
F F R R T I Q K N L H P T Y S
70
I
K
T
D
R
90
I
N
V
Q
C
C
C
D-box
G
Q
80
D
Zn
L
Y
T
C
C
R F K K C I S V
100

(6) *Summary of results*

In this chapter, I show that *Xenopus* oocytes constitute a viable model system for the reconstitution of complex regulatory networks involving TR and v-ErbA. The nuclear environment of oocytes revealed novel functions of the constitutive *trans*-activation domains present in TR and v-ErbA. The results of a detailed analysis of the constitutive and ligand-enhanced *trans*-activation functions of both α and ß isoforms of TR, exhibited at a range of TREs in oocytes, are presented. Furthermore, a constitutive *trans*-activator function of v-ErbA was shown to be induced by binding to a complex response element, clone 122 TREs, and by the presence of nuclear protein extract from anterior pituitary cells at rGH_3TRE. With the exception of some additional data, the results described in this chapter have been published and a reprint of the publication is included in Appendix 1.

2. Materials and Methods

(1) *Plasmids*

RS-rTRα, RS-v-erbA, and *tk*-TREp-CAT were gifts from R. Evans (Salk Institute for Biological Studies, La Jolla, CA). The expression vectors contain rat TRα cDNA or the *gag*-v-*erbA* oncogene under the transcriptional control of the Rous sarcoma virus (RSV) long terminal repeat (LTR) (Damm *et al.*, 1989; Thompson and Evans, 1989). *tk*-TREp-CAT contains a synthetic palindromic TRE linked to a herpes simplex virus thymidine kinase-chloramphenicol acetyltransferase fusion gene (*tk*-CAT) (Damm *et al.*, 1989). The T_3-responsive CAT reporter constructs were gifts from P. J. Romaniuk (University of Victoria, Victoria, BC, Canada) and contain TREs from the promoter regions of the rat malic enzyme gene (malTRE), the chicken lysozyme gene (lysTRE), the third intron of the rat growth hormone gene (rGH_3TRE), and the synthetic palindromic TRE (TREp) linked to a mouse mammary tumor virus (ΔMTV) LTR-CAT fusion gene (ΔMTV-CAT) (Nelson *et al.*, 1993). The pTPT-CAT reporter construct was a gift from R. N. Eisenman (Fred Hutchinson Cancer Research Center, Seattle, WA) and contains a 450 bp *Sau*3A-*Bam*HI fragment from clone 122, spanning all three TREs, cloned into pBLCAT3 (Bigler and Eisenman, 1994). pRSV-Pit-1 was a gift from H. H. Samuels (New York University Medical Center, New York, NY) and contains a cDNA clone of the rat Pit-1/GHF-1 gene linked to the RSV LTR (Fox *et al.*, 1990). The RSh-TRß construct contains the wild type human TRß1 gene, and the RSh-TRß C122>A expression plasmid contains an *in vitro*-generated mutant human TRß1 gene (alanine instead of cysteine at position 122), both under control of the RSV LTR (Nelson *et al.*, 1993). The RSh-

RXRα expression vector contains human retinoid X receptor α cDNA linked to the RSV LTR. ΔMTV-CAT contains a CAT reporter gene driven by the ΔMTV LTR promoter (Thompson and Evans, 1989). RSh-TRß, RSh-TRß C122>A, RSh-RXRα, and ΔMTV-CAT were gifts from P. J. Romaniuk. pRSV-*lacZ* was obtained from M. Harkey (University of Washington, Seattle, WA) and contains the *E. coli* ß-galactosidase gene under control of the RSV LTR (Gorman *et al.*, 1983).

(2) *Microinjections and ELISA*

A lobe of ovary was surgically removed from an adult female *Xenopus laevis* and processed as described (Allison *et al.*, 1991). Microinjections were performed according to published methods with modifications (Allison *et al.*, 1991, 1993; Nagl *et al.*, 1995). Stage V (Dumont, 1972) oocytes were microinjected with 20 nl plasmid DNA in TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) into the nucleus by the 'blind' injection method with the needle inserted in the center of the animal pole (Gurdon and Melton, 1981; Colman, 1984). After microinjection, oocytes were incubated in O-R2 (Appendix 2; Allison *et al.*, 1991) medium for 20-24 h at 18°C in the presence or absence of 100 nM T_3 (3,5,3'-triiodo-L-thyronine, Sigma, Chemical Co., St Louis, MO). Healthy oocytes (uniform pigment, not mottled) were homogenized singly in 200 µl 0.25 M Tris buffer, pH 7.5, and centrifuged at 10,000 x *g* in a microfuge at 4°C for 10 min. The supernatant was then centrifuged as before. This extract was used in the determination of CAT and ß-galactosidase protein expression levels by enzyme linked immunosorbant assay (ELISA) according to the manufacturer's specifications (5 Prime-3 Prime, Inc., Boulder, CO). The concentration of total protein in extracts from single oocytes was adjusted to a

concentration of 175 to 200 µg/ml by 1:1 dilution with Dilution Buffer. Microwells coated with rabbit polyclonal antibody specific to CAT protein or specific to the *E. coli* ß-galactosidase protein (ß-Gal) were incubated with extracts from microinjected oocytes, uninjected controls, and appropriate pure CAT or ß-Gal protein standards. Biotinylated secondary antibody to CAT or ß-Gal was then bound to the primary antibody-antigen complex. After washing, bound biotinylated antibody was quantitated colorimetrically by incubation with streptavidin-conjugated alkaline phosphatase and p-nitrophenyl phosphate as substrate. The wells were read at 405 nm against the reagent blank in a microtiter well reader (model EL 311, Bio-Tek Instruments, Winooski, VT). For each assay, a standard curve utilizing four pure protein standards was prepared, to ensure that CAT or ß-Gal concentrations of sample extracts fell within the linear range of the assay. All experiments were repeated two to three times with different animals since there can be variability in transcriptional activity between different batches of oocytes. The data on reporter gene expression levels were analyzed by nested analysis of variance (ANOVA) controlling for variability between females and between independent ELISA assays.

(3) *Immunoprecipitation of v-erbA protein*

Oocytes were injected into the nucleus with 2.5 ng RS-v-erbA in 20 nl TE, pH 8.0. Oocytes were then incubated in a sterile microtitre plate (5 oocytes/well) in 30 µl of O-R2 with 1 mCi/ml L-[^{35}S] methionine (1000 Ci/mmol, Amersham Pty Ltd., Auckland, New Zealand) at 18°C for 20-24 h. Twenty microliters of preswollen protein G-Sepharose beads (Gamma Bind Plus Sepharose, Pharmacia LKB Biotechnology, Auckland, New Zealand) in 0.45 ml NET-2 (Appendix 2; Allison *et*

al., 1991) was aliquoted per microfuge tube, followed by the addition of 30 µl of monoclonal antibody LAO38 against v-*erbA* residues 58-75 (Quality Biotech, Camden, NJ). Antibodies were bound to protein G-Sepharose beads for 2 h at room temperature with end-over-end rotation. After incubation, the resin was pelleted for 5 sec in a microfuge and resuspended in 1 ml of NET-2, and the wash was repeated three times. Nuclear and cytoplasmic fractions of 20 v-*erbA*-injected oocytes per sample were prepared manually as described (Allison *et al.*, 1991) after fixation in 1% ice-cold trichloroacetic acid for 5 min. 0.5 ml NET-2 with 0.1 mM phenylmethylsulfonyl fluoride (PMSF) was added to both fractions. Twenty uninjected control oocytes were pooled per sample and homogenized in 0.5 ml NET-2 with 0.1 mM PMSF added. Cytoplasmic fractions and control homogenates were precleared of yolk and pigment by microfuging for 10 min at 10,000 x g at 4°C. The supernatants of control samples and cytoplasmic fractions, and the nuclear fraction samples, were added to each washed protein G-Sepharose-antibody pellet and incubated at 4°C for 1 h with end-over-end rotation. After incubation, the resin with bound antigen was pelleted, and washed four times with NET-2. Forty microliters of 2 X SDS sample buffer (4% SDS, 20% glycerol, 120 mM Tris, pH 6.8, 0.01% bromophenol blue) were added to each pellet, the samples were heat denatured to release the antigen and the resin was pelleted for 5 sec in a microfuge. The supernatant, with 1 mM dithiothreitol (DTT) added, was resolved by 12% SDS-polyacrylamide gel electrophoresis followed by fluorography.

(4) *ELISA for total T_3*

Ten oocytes per sample, either incubated in 100 nM T_3 for 20 h and subsequently

washed three times in O-R2, or cultured in O-R2 only, were homogenized in 100µl 0.25 M Tris buffer, pH 7.5, and centrifuged at 10,000 x *g* in a microfuge at 4°C for 10 min. The supernatant was then centrifuged as before. This extract was used in the quantitative determination of total T_3, both bound and free hormone, in oocytes by ELISA (Enzymun-Test T_3, Boehringer Mannheim, Auckland, New Zealand). Test tubes coated with streptavidin were incubated with oocyte extracts or T_3 standards, together with anti-T_3 antibody-horseradish peroxidase (POD) conjugate. T_3 standards were prepared in 0.25 M Tris buffer, pH 7.5, at 0, 0.1, 1, 10 and 100 nM. Binding of sample T_3 to anti-T_3 antibodies was then competed by addition of biotinylated T_3 polyhapten, which anchors previously unbound anti-T_3 antibodies to the streptavidin-coated test tube wall. After washing, bound anti-T_3 antibody-POD conjugate was quantitated colorimetrically by incubation with 2, 2′-azino-di-[3-ethyl-benzthiazoline sulfonate (6)] (ABTS)/H_2O_2. The samples were transferred to micro-cuvettes and read against buffer at 420 nm in a spectrophotometer. For each assay, a standard curve utilizing the five T_3 standards was prepared. Two independent determinations were carried out with oocytes from two different animals, and four replicates (10 oocytes/replicate) were assayed in each determination.

(5) *Preparation of nuclear protein extracts from ovine anterior pituitary cells and cytoplasmic injection*

Nuclear protein extracts were prepared essentially as described by Maxson *et al.* (1988). All procedures were carried out at 4°C. Ten milliliters of sheep anterior pituitary cells maintained in primary cell culture (4.8 x 10^6 viable cells/ml) were concentrated by centrifugation, washed in phosphate-buffered saline, and then washed

once in 1.5 M glucose. The resultant partially lysed cells were homogenized gently in 10 volumes of low-salt buffer (4 mM magnesium acetate, 50 mM Tris, pH 7.4, 0.5% Nonidet P-40, 10 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride [PMSF]). Two to five strokes in a Dounce glass homogenizer were sufficient to complete lysis. The homogenate was overlaid (15 ml per tube) on step sucrose gradients consisting of 5 ml of 1.7 M sucrose on 5 ml of 2.2 M sucrose in 30-ml Nalgene tubes. The gradients were centrifuged at 5,000 *x g* for 15 min, and the gray nuclei were collected from the interface between the two sucrose solutions. The nuclei were diluted twofold with a buffer containing 75 mM NaCl, 25 mM EDTA, 5 mM Tris, pH 7.5, 20 mM 2-mercaptoethanol, and 0.1 mM PMSF. Partially lysed nuclei and chromatin were collected by centrifugation at 10,000 *x g* for 30 min. The chromatin-nuclei pellet was suspended in extraction buffer (0.45 M NaCl, 20 mM Tris, pH 7.0, 1 mM EDTA, 1 mM EGTA, 0.5 mM dithiothreitol [DTT], 0.1 mM PMSF), and vortexed for several minutes, and the insoluble material was collected by centrifugation at 10,000 *x g* for 30 min. The supernatant was set aside, and the extraction and centrifugation were repeated on the residual nuclear material with extraction buffer containing 1 M NaCl and then 2 M NaCl. The extracts of all three salt washes were pooled and concentrated by precipitation with ammonium sulfate (0.35 g/ml), dialyzed for 24 h against several changes of injection buffer (100 mM NaCl, 20 mM Tris, pH 7.0, 5 mM $MgCl_2$, 1 mM EDTA, 0.1 mM EGTA, 5% glycerol, 0.5 mM DTT, 0.1 mM PMSF), and concentrated severalfold by centrifugation in centricon 10 microconcentrators (Amicon, Beverly, MA). Extracts were stored frozen at -80°C. Five hours before microinjection of gene templates, oocytes were microinjected with 20 nl of extract into the cytoplasm.

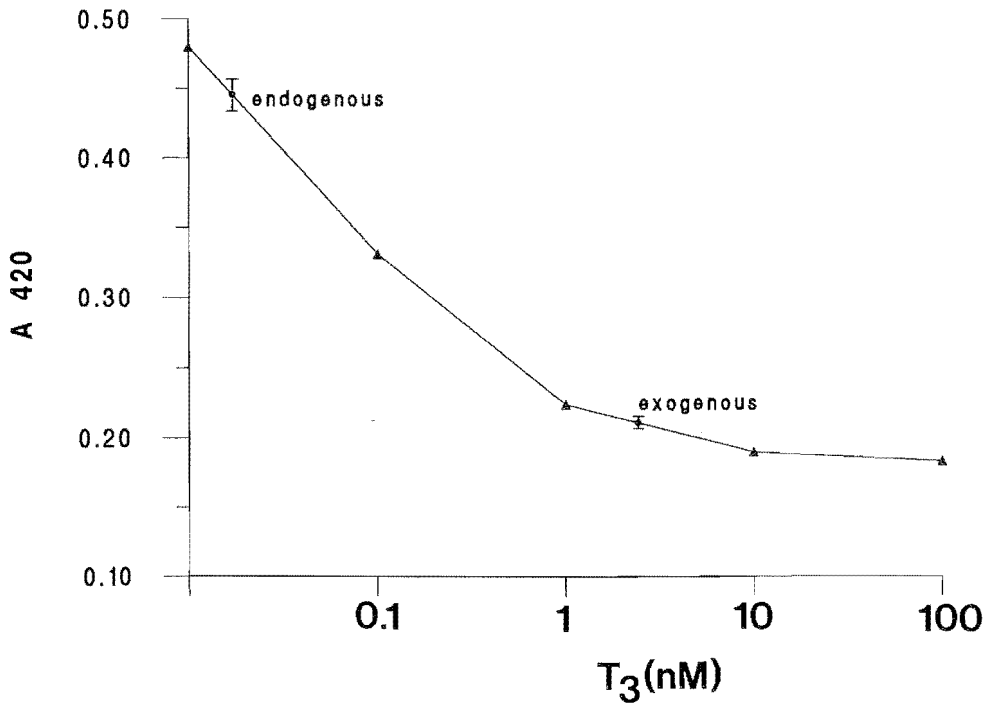
3. Results

(1) *Both constitutive and T_3-mediated gene activation functions of TRα and TRβ were induced in Xenopus oocytes and enhanced by RXR*

I was interested to establish whether *Xenopus* oocytes can be used for the reconstitution of nuclear receptor networks *in vivo*. In this context, I wished to ascertain whether oocytes provide a cellular background for nuclear hormone receptor action similar to either yeast or mammalian cells, or whether they constitute a unique context. TR mRNA is present at all stages of oogenesis (Kawahara *et al.*, 1991) and in fertilized eggs (Banker *et al.*, 1991); however, at the onset of my study, TR protein had not been detected by immunoprecipitation or Western immunoblotting (Banker *et al.*, 1991), suggesting that maternal transcripts are stored for use later in embryogenesis. Results obtained by enzyme linked immunosorbant assay (ELISA) showed that oocytes contain no detectable endogenous T_3 (< 0.1 nM). After incubation in 100 nM T_3, oocytes exhibited an intracellular hormone concentration of ~ 5 nM (Fig. 7), which falls within the physiological range for T_3 action. Thus, I presumed that *Xenopus* oocytes would be suitable for experiments investigating transcriptional regulation by exogenous TR and v-*erbA* protein at a range of diverse TREs, both with and without added T_3.

I sought to reflect the natural diversity of TREs by employing four positively regulated response elements possessing different half-site characteristics; i. e., the direct repeat TRE in the rat malic enzyme gene promoter (malTRE) (Petty *et al.*, 1990), the inverted palindromic TRE in the promoter region of the chicken lysozyme gene (lysTRE) (Baniahmad *et al.*, 1990), the tripartite TRE in the third intron of the

Figure 7. Determination of endogenous and exogenous T_3 in *Xenopus* oocytes by ELISA. A standard curve from ELISA of T_3 standards at the indicated concentrations was plotted against absorbance at 420 nm. The absorbance readings from ELISA of 10 pooled oocytes, cultured in O-R2 (endogenous) or incubated in 100 nM T_3 (exogenous) for 20 h were plotted on the standard curve to determine the T_3 content. The error bars indicate the SEMs of two independent determinations with oocytes from different females (4 replicates/treatment/determination).


0.50
0.40
0.30
0.20
0.10
A 420
endogenous
exogenous
0.1
1
10
100
T3(nM)

rat growth hormone gene (rGH_3TRE) (Sap *et al.*, 1990) and a synthetic palindromic TRE (TREp) (Damm *et al.*, 1989); and one complex response element which is strongly induced by unliganded TR and repressed by T_3 in mammalian cells (clone 122 TREs) (Bigler and Eisenman, 1994). A rat TRα (rTRα) expression plasmid and a CAT reporter gene under the control of one of five different TREs were microinjected into the oocyte nucleus. Unliganded rTRα induced transcription of all five TRE-regulated CAT reporter genes (Fig. 8). Constitutive reporter gene induction by rTRα was strongest when under the control of lysTRE, TREp, or the clone 122 TREs. CAT reporter gene transcription under control of three positively regulated TREs (malTRE, lysTRE, TREp) was further enhanced by the addition of T_3, but was unresponsive to hormone with rGH_3TRE (Fig. 8). In contrast to results obtained in CV1 (monkey kidney) cells (Bigler and Eisenman, 1994), addition of T_3 did not significantly repress TR-mediated transcription regulated by the clone 122 TREs (Fig. 8). Reporter gene induction by TR was shown to be dependent on the presence of a TRE in oocytes, as expression levels of the ΔMTV-CAT reporter gene lacking a TRE sequence were not induced by coinjection of rTRα, in the presence or absence of T_3 (Fig. 8).

Unexpectedly, there was a weak T_3-dependent induction of the *tk*-TREp-CAT and TREp-ΔMTV-CAT reporter constructs in the absence of exogenous TR, while the ΔMTV promoter alone was not responsive to T_3 (Fig. 8). Significant induction from TREp in response to T_3 was observed in samples analyzed 4 h after microinjection of the reporter gene into the nucleus and persisted over a 36 h period of hormone incubation (Fig. 9). Contrary to my original assumptions, these results suggested the presence of low levels of an endogenous TR-like activity in oocytes. A recent study

Figure 8. TR-mediated TRE-CAT reporter gene transcription in *Xenopus* oocytes. Two ng ΔMTV-CAT or *tk*-CAT reporter gene construct linked to one of five different TREs as indicated, or 2 ng ΔMTV-CAT lacking a TRE, was microinjected into the oocyte nucleus together with 0.5 ng of the rTRα expression vector. Oocytes were cultured in the presence or absence of 100 nM T_3 (TR/+T3 and TR/-T3) for 24 h. Alternatively, oocytes were microinjected with reporter constructs alone, and incubated with and without hormone (+T3/-T3). ΔMTV-TREp-CAT was tested with and without T_3 only. Activation of the reporter gene was measured in terms of the levels of CAT protein present in oocytes, analyzed individually by ELISA. Basal transcription levels of the reporter constructs in the absence of exogenous TR and T_3 were arbitrarily assigned the value 1. For each CAT reporter construct 2 or 3 independent experiments (5 oocytes per treatment) were performed and the mean fold induction over basal transcription was calculated. The error bars indicate the SEMs. Differences in levels of reporter gene transcription were significant at $p < 0.0001$.

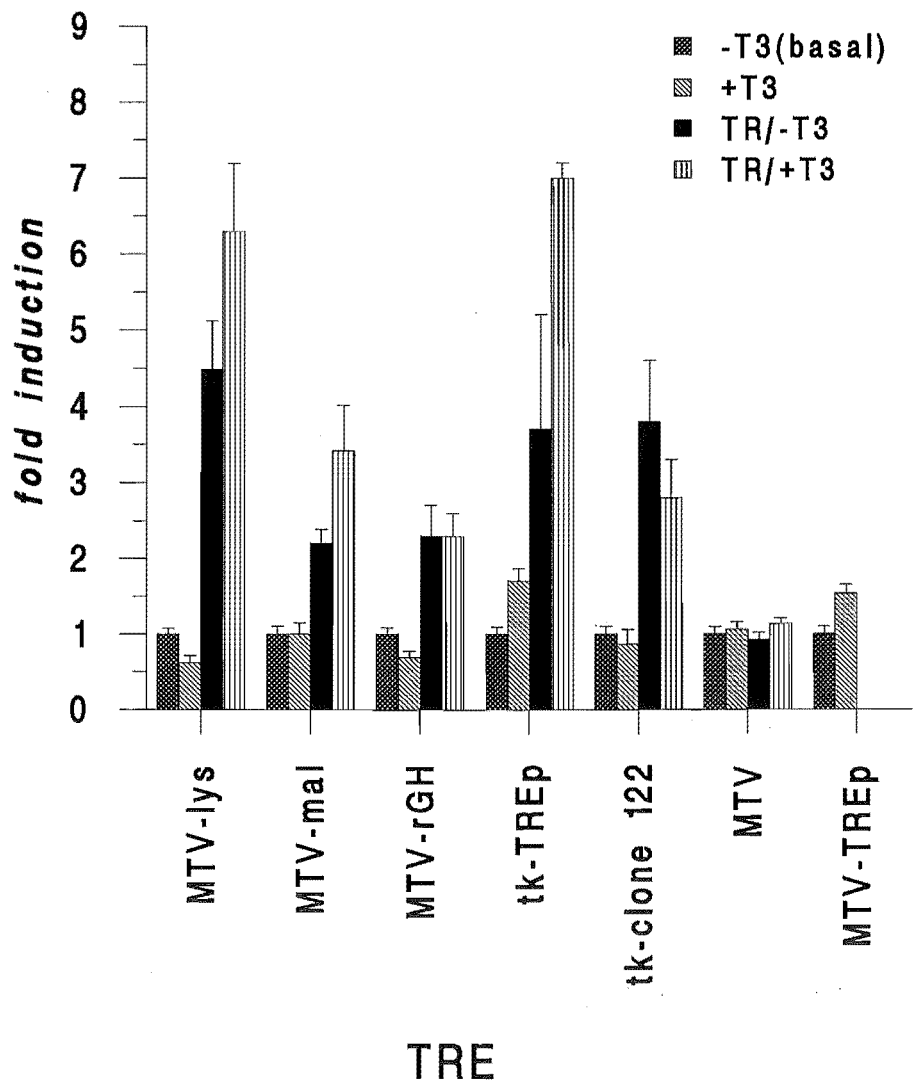
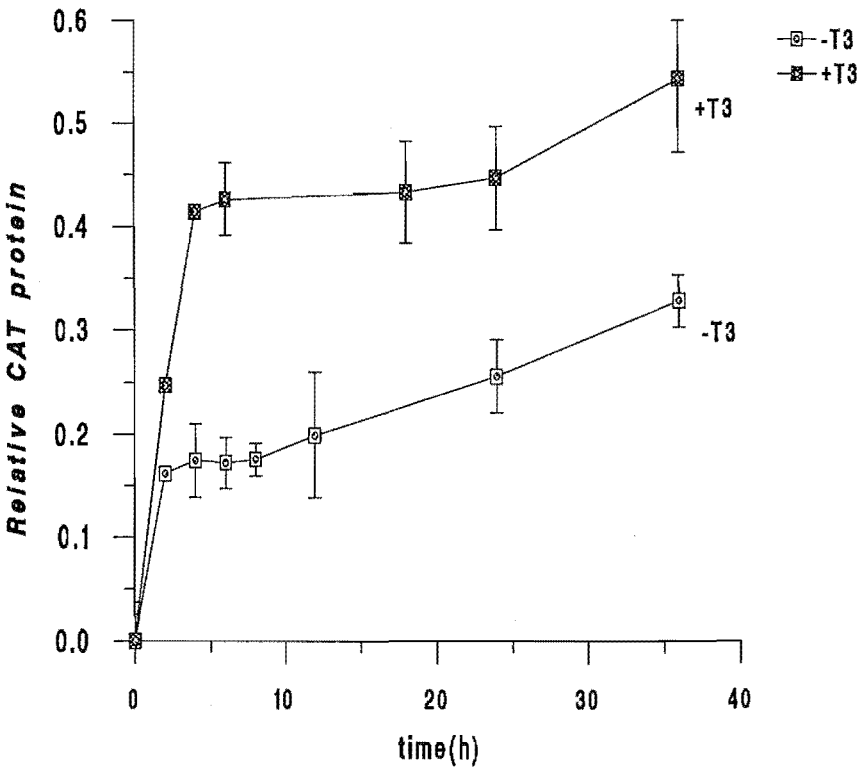

fold induction
9
8
7
6
5
4
3
2
1
0
-T3(basal)
+T3
TR/-T3
TR/+T3
MTV-lys
MTV-mal
MTV-rGH
tk-TREp
tk-clone 122
MTV
MTV-TREp
TRE

Figure 9. Time course of transcriptional activation of the *tk*-TREp-CAT reporter construct by endogenous TR in the presence of T_3. Two ng *tk*-TREp-CAT reporter construct was microinjected into the oocyte nucleus. Oocytes were then cultured with and without 100 nM T_3. Activation of the reporter gene was measured in terms of the levels of CAT protein present in single oocytes at the indicated times after microinjection, and expressed in arbitrary units based on the measurement of CAT levels by ELISA. Five oocytes per time point were assayed and the time course was repeated 4 times. The error bars indicate the SEMs.

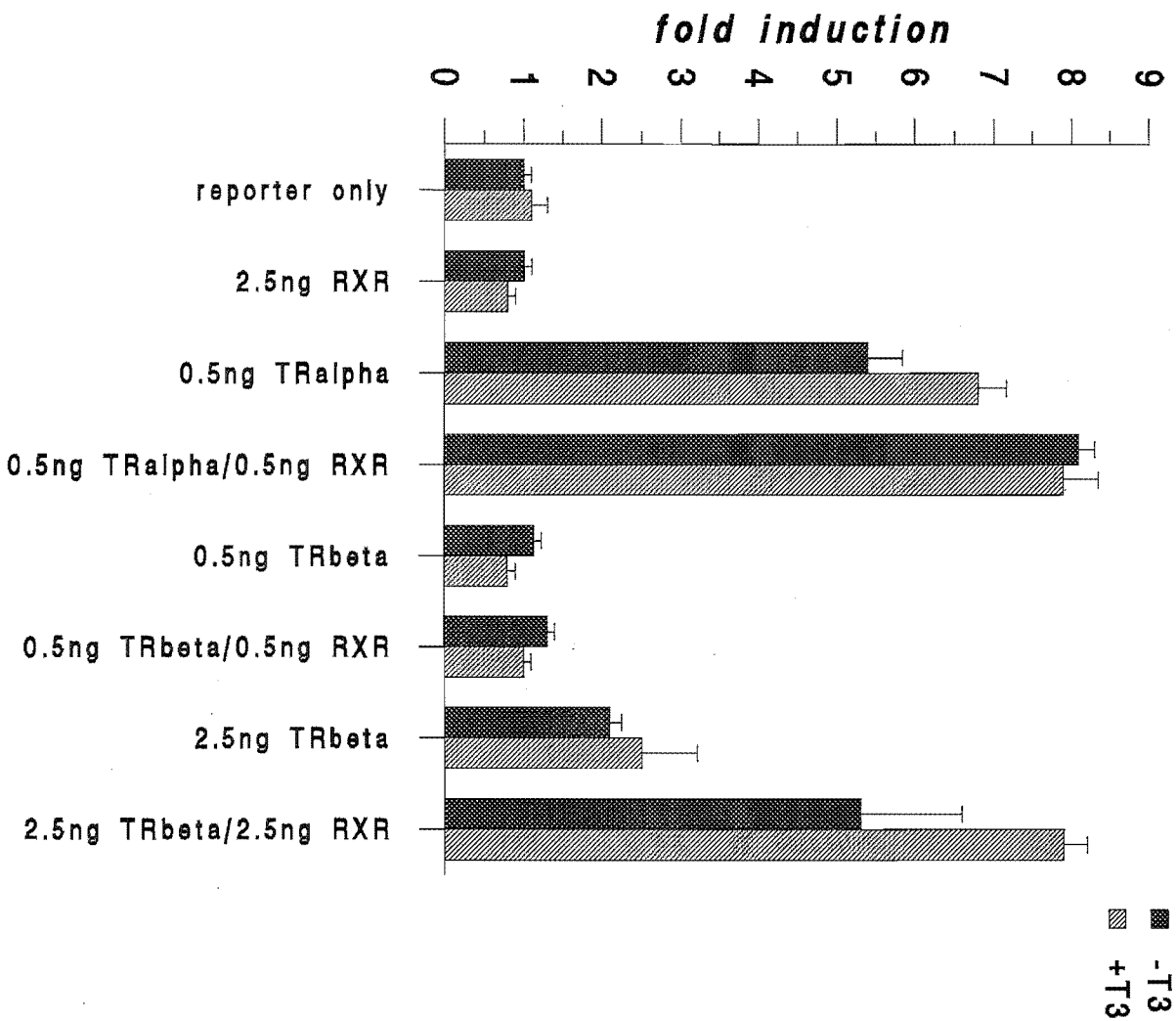

0.6
0.5
0.4
0.3
0.2
0.1
0.0
Relative CAT protein
0
10
20
30
40
time(h)
-T3
+T3
+T3
-T3

has now reported that *Xenopus* oocytes contain both TRα and TRß protein (Eliceiri and Brown, 1994). The observed T_3-mediated induction of a CAT reporter gene under control of TREp in the absence of exogenous TR indicates that these endogenous TRs can function as T_3-regulated activators.

The ß isoform of the receptor, human TRß (hTRß), also constitutively induced transcription at TREp, albeit to a lesser degree than rTRα (Fig. 10). Gene activation by hTRß was not significantly enhanced in the presence of hormone at TREp. Moreover, to achieve detectable levels of reporter gene induction by hTRß, a five times greater amount of the expression vector (RSh-TRß) had to be microinjected compared to RS-rTRα. This increased requirement might have been due to differences in the expression of the two constructs in oocytes, or to the decreased binding affinity of TRß for palindromic TREs (Privalsky *et al.*, 1990; Holloway *et al.*, 1990).

RXRs have recently been shown to heterodimerize with TRs, enhancing TR binding to TRE elements, and augmenting T_3-mediated transcriptional activation (reviewed in Mangelsdorf and Evans, 1992). Therefore, it was of interest to test how heterodimerization with RXR affects the action of TR in oocytes. In this series of experiments, equimolar amounts of expression plasmids for human RXRα (hRXRα) and rTRα, or hTRß, were microinjected into the oocyte nucleus, together with the *tk*-TREp-CAT reporter construct. Employing TREp in this experiment allowed a direct comparison between activation by both TR and RXR with induction by TR alone, because TREp binds both TR monomers and homodimers, as well as RXR/TR heterodimers (Forman *et al.*, 1992, 1995b). As shown in Fig. 10, hRXRα enhanced constitutive reporter gene activation by both rTRα and hTRß.

Figure 10. *tk*-TREp-CAT reporter gene transcription in the presence of hRXRα and rTRα or hTRß. Oocytes were microinjected with 2 ng *tk*-TREp-CAT only (reporter only), or together with varying amounts of hRXRα (RXR), rTRα (TRalpha) and hTRß (TRbeta) expression plasmids as indicated. Oocytes were incubated with and without hormone (+T3, -T3) for 24 h, and then analyzed individually by ELISA as described in Figure 8.


fold induction
0
1
2
3
4
5
6
7
8
9
reporter only
2.5ng RXR
0.5ng TRalpha
0.5ng TRalpha/0.5ng RXR
0.5ng TRbeta
0.5ng TRbeta/0.5ng RXR
2.5ng TRbeta
2.5ng TRbeta/2.5ng RXR
-T3
+T3

Coexpression of hRXRα increased T_3-independent and T_3-mediated reporter gene activation by rTRα to the same level. The presence of hRXRα enhanced the constitutive activator function of hTRß (2.5 ng expression vector microinjected) to levels seen with unliganded rTRα alone (0.5 ng expression vector). In addition, with hRXRα present, hTRß activated the reporter gene in a T_3-responsive manner to the same level as rTRα.

To summarize, both rTRα and hTRß constitutively activated reporter gene transcription at five different TREs, while activation was further inducible by T_3 at a subset of TREs. Furthermore, coexpression of hRXRα enhanced both ligand-independent and ligand-dependent activation by rTRα and hTRß at TREp. An endogenous TR activity mediated weak T_3-induction of TREp.

(2) *Only certain naturally occurring TREs acted as control elements for repression by v-ErbA in the presence or absence of T_3*

In mammalian and avian cells, TR activities are blocked by v-ErbA acting as a dominant repressor at the level of the TRE. However, this function of v-ErbA is not universal, but depends on cellular context (Privalsky *et al.*, 1990; Smit-McBride and Privalsky, 1993). Potent suppression of basal transcription of T_3-regulated genes in the absence of TR and T_3 has also been shown in mammalian cells (Damm *et al.*, 1989; Damm and Evans, 1993), and has been implicated in v-*erbA* oncogenesis (Fuerstenberg *et al.*, 1992; Privalsky, 1992; Sharif and Privalsky, 1991; Zenke *et al.*, 1990). I sought to establish the action of v-ErbA in *Xenopus* oocytes in two ways. The first series of experiments tested the competitive interaction between v-ErbA and rTRα at naturally occurring TREs, by determining the extent to which v-ErbA was

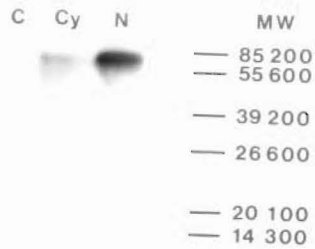
able to dominantly repress TR-induced activation of the reporter gene constructs. The second set of experiments tested the ability of v-ErbA to suppress basal promoter activity of the reporter genes in the absence of TR and T_3.

Prior to experiments, I determined whether the v-*erbA* product is transcribed and translated in *Xenopus* oocytes, and whether it is targeted to the nucleus. Nuclear and cytoplasmic fractions were prepared manually for immunoprecipitation assays. The v-*erbA* expression plasmid was shown to direct the synthesis of a 75 kDa polypeptide which was predominantly recovered from the nuclear fraction using a monoclonal anti-*v-erbA* antibody (Fig. 11). This polypeptide is identical in size to that expected for the *gag*-v-*erbA* protein (Privalsky, 1992). This indicates that a full-length v-*erbA* product is synthesized in oocytes. Furthermore, the immunoprecipitation assays showed that the v-*erbA* product is correctly targeted to the nucleus in oocytes.

Having determined that v-*erbA* is expressed in *Xenopus* oocytes, I then carried out a series of coinjection experiments to test the dominant repressor function of v-ErbA. In both the presence and absence of T_3, v-ErbA acted as a dominant repressor of rTRα-mediated CAT reporter gene transcription from lysTRE, malTRE, and TREp (Fig. 12A). Repression was most complete at TREp. The dominant action of v-ErbA was dose-dependent, as a 5-fold excess of the v-*erbA* expression plasmid produced an even greater repression of TR-mediated induction of the reporter genes than an equal ratio of v-*erbA* and TR templates (data not shown). In contrast, v-ErbA did not repress rTRα-mediated transcription under the control of rGH_3TRE (Fig. 12A). However, coexpression of the cofactor hRXRα reduced transcription in the presence of rTRα and v-ErbA at this TRE (Fig. 12A).

In mammalian cells, v-*erbA* represses basal transcription of T_3-responsive genes in

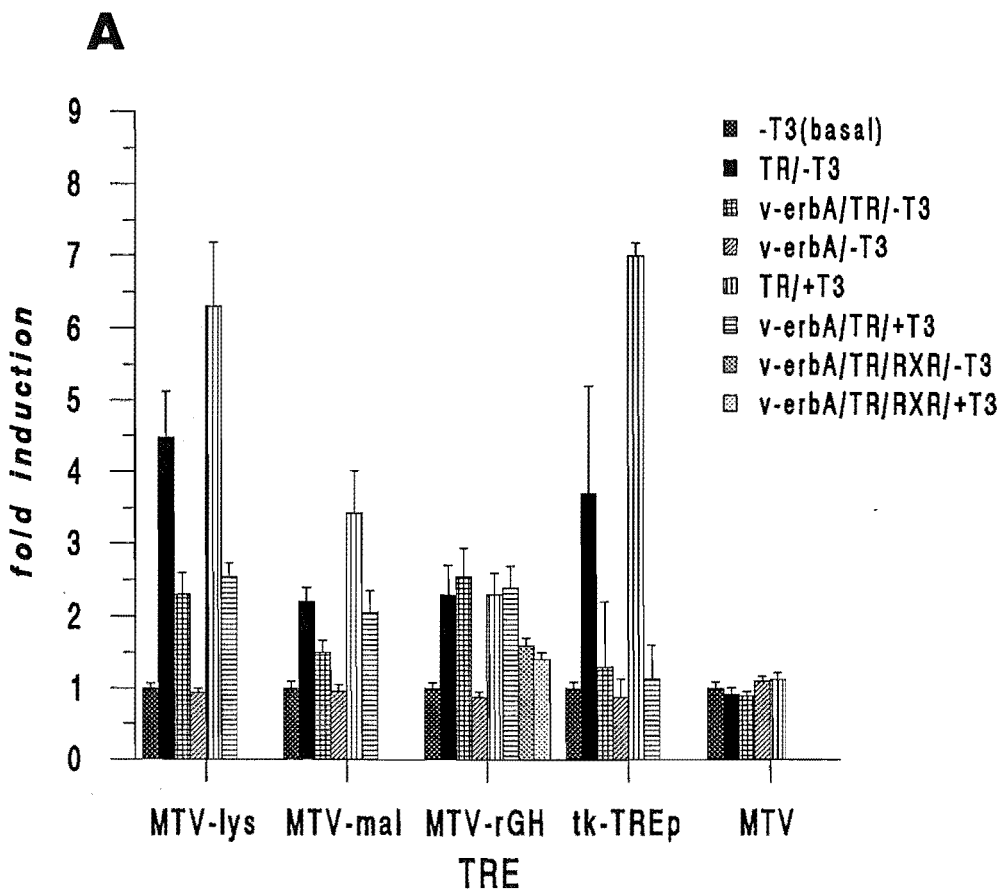
Figure 11. Immunoprecipitation of v-*erbA* protein from cytoplasmic and nuclear fractions. Oocytes microinjected with 2.5 ng v-*erbA* expression vector, and uninjected control oocytes, were cultured in O-R2 medium with 1 mCi/ml L-[^{35}S] methionine for 20 to 24 h. Nuclei of v-*erbA*-injected oocytes were then manually isolated. Cytoplasmic (Cy) and nuclear (N) fractions of 20 v-*erbA*-injected oocytes, and homogenates of 20 uninjected whole oocytes (C), were incubated with protein G-Sepharose-antibody complexes in an immunoprecipitation assay. Labelled polypeptides were recovered and separated electophoretically on 12% SDS-polyacrylamide gels followed by fluorography. The 75 kDa *gag*-v-*erbA* protein (Privalsky, 1992) was predominantly immunoprecipitated from the nuclear fraction.


C
Cy
N
MW
85 200
55 600
39 200
26 600
20 100
14 300

the absence of T_3 (Damm *et al.*, 1989; Damm and Evans, 1993). In the present study, v-ErbA did not act as a repressor of basal gene transcription under control of four different positively regulated TREs in oocytes (Fig. 12A). Repression of basal transcription by v-ErbA would have been detectable by the CAT ELISA employed, since the linear range of the assay (tested down to A_{405} ~0.15) extended below the CAT protein levels observed due to basal promoter activity (A_{405} ~0.23-0.3) (data not shown). The loss of this function by v-ErbA parallels the lack of repression of basal transcription by unliganded exogenous TR in oocytes. Importantly, expression of v-*erbA* in oocytes abolishes its function as a repressor of basal promoter activity, but does not interfere with its dominant repressor potential.

I showed that dominant repression by v-ErbA in the oocyte is a specific effect in the following ways. First, transcription levels of the ΔMTV-CAT reporter plasmid were unaffected by the presence of rTRα and/or v-ErbA with and without T_3, showing that gene regulation by rTRα and v-ErbA is dependent on the presence of a TRE (Fig. 12A). Second, T_3-dependent induction by rTRα of the ΔMTV-lysTRE-CAT reporter gene was unaffected by the coinjection of the *lacZ* expression vector in place of the v-*erbA* construct (Fig. 12B). Both in the presence and absence of the *lacZ* vector, T_3 enhanced reporter gene expression 1.5 fold over levels induced by unliganded rTRα. This control was performed at a 2-fold greater concentration of the *lacZ* vector (5 ng) compared with experiments using v-*erbA* construct (2.5 ng). Dominant repression by v-ErbA was thereby shown not to be caused by 'non-specific squelching' (Ptashne, 1988); i.e., essential transcription factors are not simply being titrated by the presence of an excess of v-*erbA* expression template.

Figure 12. Effects of v-ErbA on TRE-CAT reporter gene transcription. (*A*) Dominant repression of TR-mediated TRE-CAT reporter gene transcription by v-ErbA in *Xenopus* oocytes. Oocytes were cultured in the presence or absence of 100 nM T_3 after microinjection with 2 ng ΔMTV-CAT or *tk*-CAT reporter gene construct linked to four different TREs as indicated, or 2 ng ΔMTV-CAT without a TRE, and 0.5 ng rTRα expression vector alone (TR/+T3, TR/-T3), or together with a 5-fold excess of v-*erbA* (2.5 ng) (v-erbA/TR/+T3, v-erbA/TR/-T3). Two ng rGH_3TRE reporter construct plus 0.5 ng rTRα, 2.5 ng v-*erbA*, and 2.5 ng hRXRα expression plasmids were also tested (v-erbA/TR/RXR/-T3, v-erbA/TR/RXR/+T3). In addition, 2 ng TRE-CAT reporter gene construct was microinjected into the oocyte nucleus with 2.5 ng v-*erbA* expression plasmid, and oocytes were cultured without T_3 (v-erbA/-T3). Alternatively, oocytes were microinjected with reporter constructs alone, and incubated in the absence of T_3 (-T3). After 24 h, oocytes were assayed individually for CAT protein by ELISA. Basal transcription rates of the CAT reporter constructs in the absence of exogenous TR, v-ErbA and T_3 (-T3) were arbitrarily assigned the value 1. Two or 3 independent experiments (5 oocytes per treatment) were performed for each TRE-CAT reporter gene construct and the mean fold induction over basal transcription was calculated. The error bars indicate the SEMs. Differences in levels of reporter gene transcription were significant at $p < 0.0001$. (*B*) T_3-induction of lysTRE-CAT reporter gene transcription in the presence of rTRα and *lacZ* expression plasmids. Oocytes were microinjected with 2 ng ΔMTV-lysTRE-CAT reporter gene construct plus 0.5 ng rTRα with or without 5 ng *lacZ* expression vector, as indicated, and incubated in the presence or absence of 100 nM T_3 (-T3/+T3). A single experiment with 5 oocytes per treatment was performed and analyzed as described for (*A*). (*C*) Constitutive *trans*-activation by v-ErbA at the clone 122 TREs. Oocytes were cultured with and without T_3 after microinjection of 2 ng pTPT-CAT plasmid, containing the clone 122 TREs linked to a *tk*-CAT reporter construct (tk-clone 122), or 2 ng ΔMTV-lysTRE-CAT plasmid, and 0.5 ng v-*erbA* expression vector (v-erbA/+T3, v-erbA/-T3). Alternatively, oocytes were microinjected with reporter constructs alone, and incubated in the absence of T_3 (-T3). Three independent experiments with 5 oocytes per treatment were performed and analyzed as described for (*A*).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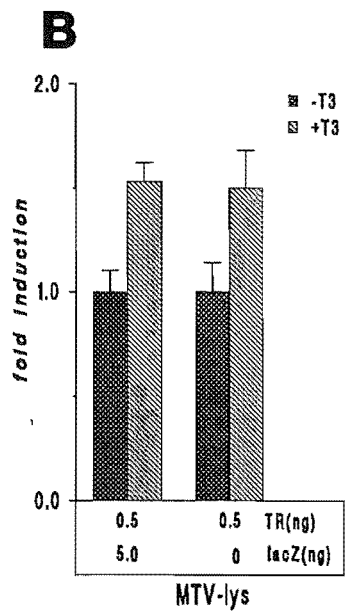
-T3(basal)
TR/-T3
v-erbA/TR/-T3
v-erbA/-T3
TR/+T3
v-erbA/TR/+T3
v-erbA/TR/RXR/-T3
v-erbA/TR/RXR/+T3
MTV-lys
MTV-mal
MTV-rGH
tk-TREp
MTV
TRE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2.0
-T3
+T3
0.5
0.5
TR(ng)
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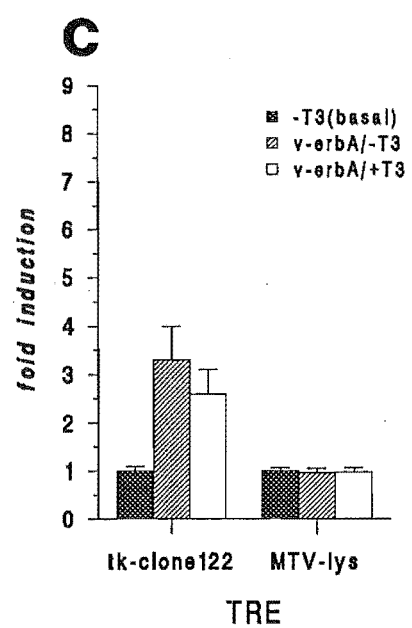
lacZ(ng)
MTV-lys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7
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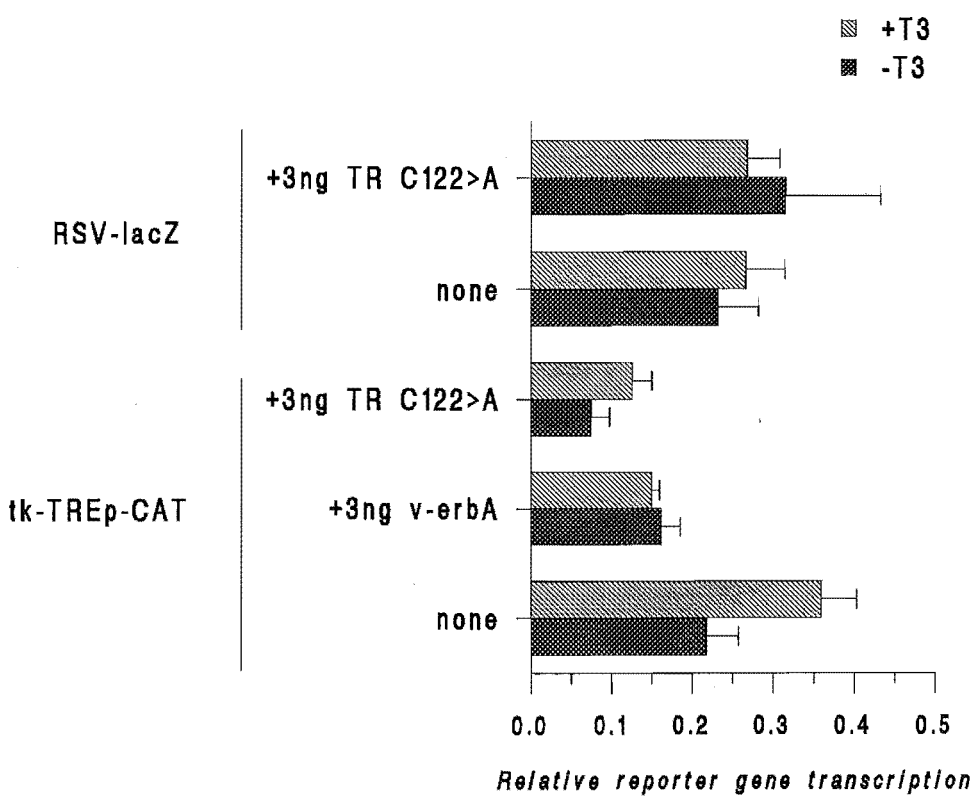
9
-T3(basal)
v-erbA/-T3
v-erbA/+T3
tk-clone122
MTV-lys
TRE

The v-*erbA* protein binds T_3 only at very low affinity, due to several mutations in the C-terminal domain (Damm *et al.*, 1989; Forrest *et al.*, 1990; Privalsky *et al.*, 1988; Sap *et al.*, 1989). Presumably, this lack of hormone binding would render v-ErbA highly resistant to undergoing a ligand-induced conformational change. For this reason, I was interested to ascertain how v-ErbA acts at the clone 122 TREs in oocytes. In mammalian cells, T_3 inhibits the activator function and induces the repressor function of TR at this TRE (Bigler and Eisenman, 1994). I showed in the previous section that, in oocytes, TR constitutively activated transcription at the clone 122 TREs. I also observed constitutive *trans*-activation of the reporter gene construct by v-ErbA (Fig. 12C). This induction by v-ErbA was not significantly affected by T_3. The v-*erbA* protein was also unresponsive to T_3 at lysTRE in oocytes (Fig. 12C).

In the previous section I reported that there was a weak T_3-dependent induction of the TREp-CAT reporter gene in the absence of exogenous TR, suggesting the presence of low levels of T_3-regulated endogenous TR in oocytes. The T_3-dependent reporter gene induction by endogenous TR was dominantly repressed to the same level by v-ErbA and a dominant negative *in vitro*-generated mutant of human TRß (TR C122>A) (Fig. 13). This DNA-binding deficient mutant is thought to repress TR-mediated gene transcription by forming inactive dimers with TR (Nelson *et al.*, 1993). Therefore, dominant repression by TR C122>A suggests that endogenous TR was able to dimerize with the exogenous mutant TRß. The slight T_3-dependence of transcription in the presence of TR C122>A was interpreted as not biologically significant, because of high sample variability. Repression of the reporter gene by v-ErbA or TR C122>A was dependent on the presence of a TRE. The *lacZ* reporter gene, which lacks a TRE, was expressed at levels similar to those of the CAT

Figure 13. Dominant repression of an endogenous TR-like activity by v-ErbA or an *in vitro*-generated mutant of TR. Oocytes were microinjected with 2 ng of *tk*-TREp-CAT reporter plasmid alone (none), or together with v-*erbA* or TR C122>A expression plasmids in the presence or absence of 100 nM T_3 (+T3, -T3) in the combinations indicated. As a control, 2 ng RSV-*lacZ* reporter gene instead of *tk*-TREp-CAT construct was coinjected with TR C122>A or injected alone (none). Relative reporter gene transcription was expressed in arbitrary units based on measurements of CAT or ß-galactosidase protein levels by ELISA. Two independent experiments with 5 oocytes per treatment were performed. The error bars indicate the SEMs. Transcription levels of the RSV-*lacZ* construct did not differ significantly between treatments ($p < 0.39$). Transcription levels of the *tk*-TREp-CAT construct in the presence or absence of T_3 were significantly different at $p < 0.0001$.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-T3
RSV-lacZ
+3ng TR C122>A
none
tk-TREp-CAT
+3ng TR C122>A
+3ng v-erbA
none
0.0
0.1
0.2
0.3
0.4
0.5
Relative reporter gene transcription

reporter gene, and was not repressed by either v-ErbA (data not shown) or TR C122>A (Fig. 13).

In summary, my results show that v-ErbA displays an altered pattern of activity in oocytes, compared to both mammalian cells and the yeast, *S. cerevisiae*. v-ErbA did not repress basal reporter gene transcription, yet acted as a dominant repressor of TR action at the positively regulated TREs tested, with the exception of rGH_3TRE. At the clone 122 TREs, v-ErbA acted as a constitutive activator.

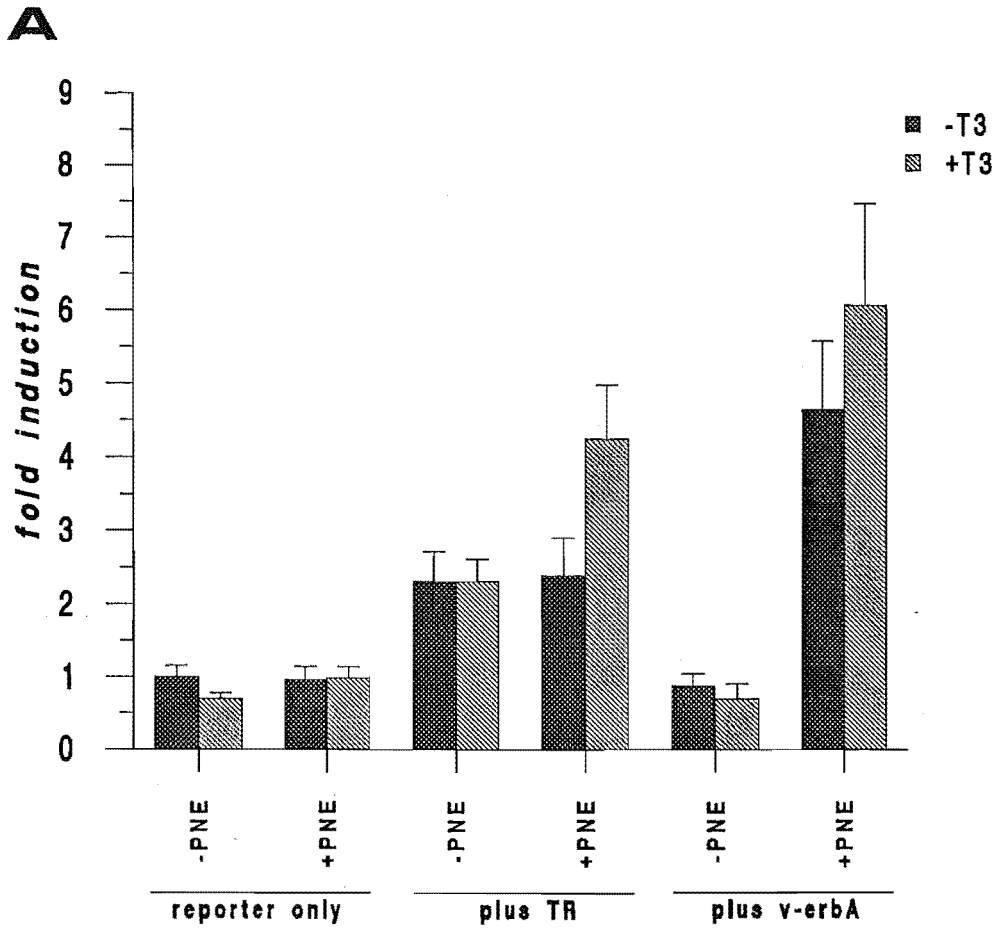
(3) *Pituitary-specific transcriptional regulation of rGH_3TRE by rTRα and v-ErbA*

In a previous section, I showed that the T_3-dependent activator function of rTRα was not induced at rGH_3TRE in the presence of hormone (Fig. 8). Previous studies have shown that optimal transcription of the rat growth hormone gene (rGH) under control of TRE sequences in the promoter region is dependent on a cell-specific factor, Pit-1/GHF-1, binding at upstream elements (Lefevre *et al.*, 1987). Since the rGH gene is expressed only in somatotrophic cells of the anterior pituitary gland, gene regulation at rGH_3TRE, the TRE from the third intron, presumably also requires cell-specific auxiliary factors, of known or unknown origin. For this reason, I sought to reconstitute the nuclear environment of anterior pituitary cells in oocytes. *Xenopus* oocytes possess the experimentally attractive feature of correctly targeting nuclear proteins, injected into the oocyte cytoplasm, to the nucleus (see, for example, Maxson *et al.*, 1988). In this experiment, nuclear protein extract from ovine anterior pituitary cells was injected into the oocyte cytoplasm five hours before nuclear microinjection of gene templates.

When oocytes were microinjected with nuclear protein extract, both the hormone-independent and hormone-dependent activator functions of rTRα were induced at rGH_3TRE (Fig. 14A). Surprisingly, under the same conditions, v-ErbA acted as a constitutive activator at rGH_3TRE, whose strength of activation exceeded that of rTRα (Fig. 14A). Increasing the amount of v-*erbA* expression template injected did not enhance this response further (data not shown). In contrast, v-ErbA did not affect basal promoter activity of this TRE-reporter construct in the absence of nuclear protein extract (Fig. 14A).

These findings suggested that auxiliary factor(s), present in the injected nuclear protein extract from anterior pituitary cells, significantly altered the effect of both rTRα and v-ErbA on reporter gene regulation at rGH_3TRE in oocytes. Two plausible candidates for this auxiliary role are the pituitary-specific transcription factor Pitl/GHF-1 and RXR. To test this possibility, expression vectors for either Pit-1/GHF-1 or hRXRα were coinjected with rTRα or v-*erbA* constructs, and reporter gene transcription regulated by rGH_3TRE was assayed. Transcription was weakly induced by hRXRα alone, while Pit-1/GHF-1 had no significant effect on basal promoter activity (Fig. 14B). The activity patterns of rTRα and v-ErbA observed in the presence of nuclear protein extract of anterior pituitary cells were not replicated in the presence of hRXRα or Pit-1/GHF-1 (Fig. 14B). Instead, coexpression of hRXRα or Pit-1, together with rTRα, reduced *trans*-activation of rGH_3TRE (Fig. 14B) compared to levels of *trans*-activation by rTRα alone (cf. Figs. 12A and 14B). Pit-1 repressed induction of rGH_3TRE by liganded rTRα below basal transcription (Fig. 14B). Compared to levels in the presence of v-ErbA only (Fig. 14A), coexpression

Figure 14. Pituitary-specific transcriptional regulation of rGH_3TRE by rTRα and v-ErbA. (*A*) Action of rTRα and v-ErbA at rGH_3TRE in the presence of pituitary nuclear extract from ovine anterior pituitary cells. Oocytes were either microinjected with 20 nl of nuclear protein extract into the cytoplasm five hours before nuclear injection of gene templates (+PNE) or microinjected with the gene templates in the absence of nuclear protein extract (-PNE). Oocytes were then cultured in the absence or presence of 100 nM T_3 (-T3, +T3) after microinjection with 2 ng ΔMTV-rGH_3TRE-CAT reporter gene construct alone (reporter only), or together with 0.5 ng rTRα (plus TR) or v-*erbA* expression vector (plus v-erbA). After 24 h, oocytes were assayed individually for CAT protein by ELISA. Basal transcription rates of the CAT reporter construct in the absence of nuclear protein extract, exogenous TR, v-ErbA and T_3 (reporter only, -PNE, -T3) were arbitrarily assigned the value 1. (*B*) Action of rTRα and v-ErbA at rGH_3TRE in the presence of hRXRα or Pit-1/GHF-1. Oocytes were microinjected with 2 ng ΔMTV-rGH_3TRE-CAT reporter gene construct only (reporter only, no co-factor), or together with 2.5 ng expression vector for either hRXRα (+RXR) or Pit-1/GHF-1 (+Pit-1). Alternatively, 0.5 ng expression plasmid for rTRα (plus TR) or v-*erbA* (plus v-erbA) were microinjected with ΔMTV-rGH_3TRE-CAT, and 2.5 ng expression vector for hRXRα (+RXR) or Pit-1/GHF-1 (+Pit-1) was added. Oocytes were cultured with and without T_3 (+T3, -T3). After 24 h, oocytes were assayed individually for CAT protein by ELISA. Basal transcription rates of the CAT reporter construct in the absence of co-factors and T_3 (reporter only, no co-factor, -T3) were arbitrarily assigned the value 1. (*A*) and (*B*) Three independent experiments (5 oocytes per treatment) were performed and the mean fold induction over basal transcription was calculated. The error bars indicate the SEMs.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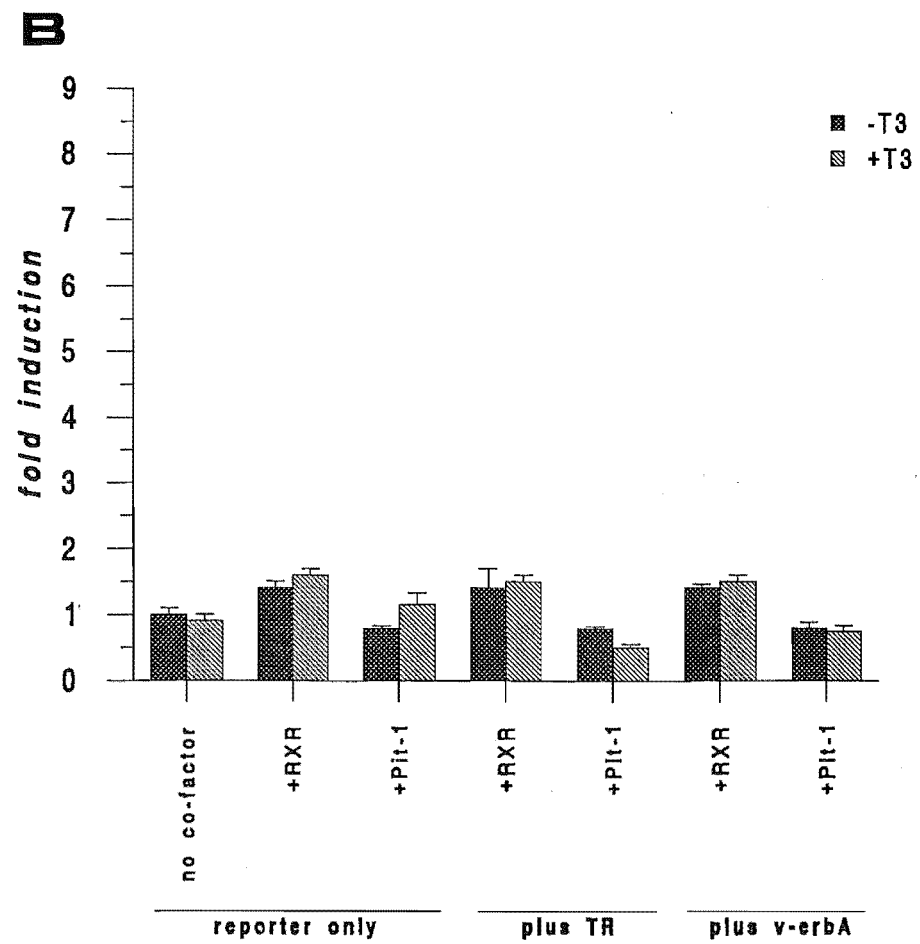
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+RXR
+Pit-1
+RXR
+Pit-1
reporter only
plus TR
plus v-erbA

of hRXRα with v-ErbA induced transcription slightly, while Pit-1 had no effect (Fig. 14B). With hRXRα present, transcription was induced equally by rTRα or v-ErbA. These effects were not further investigated, since this series of experiments was carried out to test hRXRα and Pit-1 as potential candidates for conferring positive T_3-responsiveness on rTRα, and strong constitutive activator function on v-ErbA, at rGH_3TRE. Taken together, these results suggest that auxiliary factor(s), other than RXR and Pit-1/GHF-1, present in nuclear protein extracts of anterior pituitary cells, are necessary for T_3-induction of rGH_3TRE by rTRα and conversion of v-ErbA into a constitutive activator at this TRE.

4. Discussion

(1) *Both α and ß isoforms of TR act as constitutive activators in oocytes*

In *Xenopus* oocytes, rTRα acted as a ligand-independent transcriptional activator at four positively regulated TREs (lysTRE, malTRE, TREp and rGH_3TRE). With the exception of rGH_3TRE, addition of T_3 led to a further increase in CAT reporter gene expression by rTRα. The 3.4 to 7-fold T_3-dependent reporter gene induction observed in oocytes was comparable to activation in mammalian cells (Baniahmad *et al.*, 1992; Damm *et al.*, 1989; Hermann *et al.*, 1993). hTRß also acted as a weak hormone-independent activator at TREp, but was not further inducible by hormone. The observed differences in ligand-independent activation function between rTRα and hTRß are most likely due to structural divergence in the N-terminal domain (Glass and Rosenfeld, 1991).

Both ligand-independent and ligand-induced activation functions (Barettino *et al.*, 1994; Bigler and Eisenman, 1994; Forman *et al.*, 1988; Forman and Samuels, 1990; Saatcioglu *et al.*, 1993a) exhibit distinct cell type and promoter context specificity in TR and other receptors belonging to the steroid/thyroid hormone receptor gene superfamily (Privalsky *et al.*, 1990; reviewed in Gronemeyer, 1992). Constitutive *trans*-activation by TR has also been shown in the yeast, *S. cerevisiae* (Privalsky *et al.*, 1990; Smit-McBride and Privalsky, 1993), and in *in vitro* transcription assays using extracts from NT2 embryonal carcinoma cells or Namalwa B cells (Lee *et al.* 1994a). Taken together with these results, the ligand-independent activation function of mammalian TRs in oocytes points to a possible requirement of inhibitory auxiliary factor(s) for transcriptional repression. The finding that gene activation by unliganded TR at the clone 122 TREs was not reversed by T_3 in oocytes, while being suppressed by ligand in CV1 cells (Bigler and Eisenman, 1994), also points to a requirement for an inhibitory auxiliary factor for the suppression of *trans*-activation by TR.

I have shown that hRXRα enhances both ligand-independent and ligand-induced activation functions of rTRα and hTRß at TREp, in analogy to the enhancement of both activation functions of hTRß by hRXRα seen in the yeast (Lee *et al.*, 1994b). Thus, RXRα is not the inhibitory factor in question. My results are in close agreement with recent evidence which suggests that an inhibitory factor suppresses *trans*-activation by unliganded TR in mammalian cells (Baniahmad *et al.*, 1995; Casanova *et al.*, 1994), and which also showed that RXR was not the major inhibitory factor in two cell lines tested (Casanova *et al.*, 1994). More recently, two mammalian corepressors of TR and v-ErbA, N-CoR and SMRT, were identified by

two-hybrid screening in yeast (Chen and Evans, 1995; Hörlein *et al.*, 1995; Kurokawa *et al.*, 1995). N-CoR and SMRT interact with the hinge domain of unliganded TR and v-ErbA to mediate transcriptional silencing, and are released from TR upon ligand binding. However, corepressor release by ligand occurred only at positive TREs, and it is presently unknown whether N-CoR and SMRT are involved in transcriptional repression by liganded TR at negative TREs. Thus, either N-CoR, SMRT, or a related corepressor, most likely constitutes the component missing in oocytes that is required for transcriptional repression by unliganded rTRα, hTRß and v-ErbA at positive TREs, and possibly also for repression by liganded rTRα at the clone 122 TREs.

The present study demonstrated that mammalian rTRα/hRXRα and hTRß/hRXRα heterodimers constitutively activate transcription from a palindromic TRE linked to the viral *tk* promoter in *Xenopus* oocytes. Interestingly, heterodimers of *Xenopus* specific TRs (xTRs) and RXRs (xRXRs), expressed from microinjected mRNAs in *Xenopus* oocytes, strongly repress the T_3-inducible promoter of the *Xenopus* TRßA (xTRßA) gene in the absence of T_3, and efficiently induce transcription in response to T_3 (Wong and Shi, 1995; Wong *et al.*, 1995). The 1.6 kb xTRßA promoter sequence contains a DR4 TRE which is located within the transcribed region, several other TREs with diverse half-site characteristics in the 5' region, and extensive flanking regions (Machuca *et al.*, 1995; Ranjan *et al.*, 1994; Wong *et al.*, 1995). These elements might act in concert to mediate repression and activation by xTR/xRXR. The differential mode of action of mammalian and *Xenopus* TR/RXR heterodimers, at the TREp-*tk* and xTRßA promoters respectively, serves to highlight the importance of the chromatin environment around TRE binding sites for transcriptional regulation

by TR (Almouzni and Wolffe, 1993a; Wong and Shi, 1995; Wong *et al.*, 1995). In addition, and presumably unlike their mammalian counterparts, unliganded xTR/xRXR heterodimers might be able to directly inhibit the oocyte transcription machinery without requiring a corepressor(s).

(2) *The v-erbA protein displays a novel pattern of activity in oocytes*

Conversion of TR and v-ErbA into ligand-independent transcriptional activators has been shown to be mediated either by cell-specific protein-protein interactions (Forman *et al.*, 1988; Privalsky *et al.*, 1990), or by direct DNA binding (Saatcioglu *et al.*, 1993a; Bigler and Eisenman, 1994). A constitutive activation function has also been identified in the N-terminal region of v-ErbA (Saatcioglu *et al.*, 1993a). Under conditions that induce the constitutive activation function of TR, v-ErbA also acts as a constitutive activator in yeast (Privalsky *et al.*, 1990), and in mammalian cells (Saatcioglu *et al.*, 1993a). In contrast, I show here that the oocyte-specific cellular background induces the ligand-independent activation function of rTRα, but not of v-ErbA, at four positive TREs, suggesting that induction of this function can be differentially controlled in TRα and v-ErbA within the same cellular context. On the other hand, both rTRα and v-ErbA constitutively activated reporter gene transcription at the complex clone 122 TREs (Fig. 15). This TRE belongs to the same class of response elements as the TRE found in the Rous sarcoma virus-long terminal repeat, which mediates strong T_3-independent activation by TR and v-ErbA in HeLa cells (Saatcioglu *et al.*, 1993a). Binding to this type of TRE induces a conformational change and renders the constitutive N-terminal activation domain accessible to the transcription machinery (Saatcioglu *et al.*, 1993a). In summary, expression of TR

Figure 15. TRE-modulated action of v-ErbA in oocytes. The identity of the nucleotide residue at the fourth position of the two primary half-sites in rGH_3TRE is indicated (boxed italicized letter) (Chen *et al.*, 1993). Intervening nucleotides between half-sites are indicated by numbers. Arrows indicate orientation of half-sites. Differential levels of dominant repression are expressed in relative terms; (+++) indicates complete repression, (++) indicates moderate repression, and (+) indicates weak repression by v-ErbA.

TRE-modulated action of v-ErbA in oocytes:

1. Dominant repression

Strength of repression

TREp AGGTCA 0 TGACCT +++

malTRE GGGTTA 4 AGGACA ++

lysTRE *TGACCC 6 AGGTCA* +

2. No repression of TR function by v-ErbA alone

rGH TRE AGG[T]AA 6 AGTCCC 6 AGG[T]CA (++; RXR corequirement)

3. Activation

clone 122 TREs

TRE1 TAAGCCCCCAGCCCCCCGACATCCAGGACGCCCCAAA

TRE2 ATACCTTATTACCTCATCATGTGAAATAG

TRE3 TCCGAGTGGACTCGGCTCGGTCATTGGGTG

and v-ErbA in oocytes induced the constitutive activation function only in TR at positive TREs, whereas this function was activated in both TR and v-ErbA at the clone 122 TREs in the same cellular context. It follows that the responses of the ligand-independent activation domains of TR and v-ErbA to cell-specific and TRE-mediated induction are not equivalent.

In oocytes, v-ErbA did not repress basal promoter activity under control of all four positive TREs tested (TREp, lysTRE, malTRE, and rGH_3TRE). Like TR, the v-*erbA* protein possesses a repressor function located in the hinge domain (Damm and Evans, 1993). Transcriptional repression by TR is observed on minimal promoters containing a TATA box and TRE sequence (Baniahmad *et al.*, 1990, 1992), and cell-free transcription assays have shown that unliganded TR functions as an active repressor of T_3-responsive genes by inhibiting the formation of a functional preinitiation complex (Fondell *et al.*, 1993). Presumably, repression by TR and v-ErbA occurs by a similar mechanism, namely, by interference with general transcription factors. In oocytes, protein-protein interactions between the repressor domain of v-ErbA and the general transcription machinery might be prevented by a lack of auxiliary inhibitory factor(s) or cell-specific characteristics of the general transcription complex.

(3) *Dominant negative repression by v-ErbA depends on the nature of the TRE*

The action of v-ErbA at rGH_3TRE has not been tested in mammalian cells to my knowledge. In oocytes, v-ErbA failed to repress TR-mediated induction at rGH_3TRE. At the same time, v-ErbA dominantly repressed *trans*-activation by TR at three other positive TREs with diverse half-site characteristics (malTRE, lysTRE and TREp).

The TRE-modulated action of v-ErbA in oocytes is summarized in Fig. 15. Although both primary half-sites of rGH_3TRE possess a favorable thymidine nucleotide at the fourth position (Chen *et al.*, 1993; Sap *et al.*, 1990), and the sequence of the major binding site is identical to the AGGTC/GA consensus motif (Fig. 15), it appears that v-ErbA does not successfully compete with rTRα for binding due to the structure of rGH_3TRE. As shown by other workers, heterodimerization with RXR-related factors can be essential for the dominant negative action of v-ErbA (Chen and Privalsky, 1993; Hermann *et al.*, 1993). In the present study, a reduced level of *trans*-activation of rGH_3TRE by rTRα and v-ErbA was observed in the presence of hRXRα. However, the significance of this finding is presently unclear, since hRXRα also reduced *trans*-activation by rTRα alone at this TRE.

(4) ***An activity in nuclear protein extracts from anterior pituitary cells conferred T_3-responsiveness to rTRα and converted v-ErbA into a constitutive activator at rGH_3TRE***

My findings suggest that T_3-induction of rGH_3TRE is regulated in a cell-specific manner. In the presence of nuclear protein extract from anterior pituitary cells, the T_3-dependent activation function of rTRα was induced at rGH_3TRE in addition to T_3-independent *trans*-activation by rTRα without extract. I showed that T_3-responsiveness of rTRα was not mediated by hRXRα or the pituitary-specific transcription factor Pit-1/GHF-1 which is required for efficient T_3-induction of the growth hormone gene in somatotrophs (Lefevre *et al.*, 1987). General mammalian-type transcription factors might have been involved in conferring T_3-responsiveness to rTRα, since rGH_3TRE is also T_3-inducible in CV1 cells (Sap *et al.*, 1990). The

tripartite rGH_3TRE, although located in the third intron of the rat growth hormone gene, exhibits a markedly higher affinity for TR, as well as greater gene induction ability, than the TRE located in the promoter region of the gene, indicating an important *in vivo* role for this TRE sequence (Sap *et al.*, 1990). Further indications of the importance of regulatory regions in introns are the presence of a glucocorticoid-responsive element in the first intron of the human growth hormone gene (Slater *et al.*, 1985), and the requirement for the presence of introns in the rat growth hormone gene for efficient expression in transgenic animals (Brinster *et al.*, 1988). Interestingly, v-ErbA acted as an efficient constitutive activator at rGH_3TRE in the presence of nuclear protein extract from anterior pituitary cells, whereas it had no effect on basal promoter activity under the control of rGH_3TRE without extract. Coexpression of Pit-1/GHF-1 or hRXRα did not replicate the mode of action of the v-*erbA* protein as a constitutive activator seen with the nuclear protein extract. This finding suggests that indirect or direct interactions with an as yet unidentified nuclear factor present in pituitary cells result in a conformational change in v-ErbA at rGH_3TRE that induces its constitutive activation function. The conversion of v-ErbA into a constitutive activator by nuclear protein extract from anterior pituitary cells at rGH_3TRE suggests that the dominant negative phenotype of the v-*erbA* oncogene can be abolished by either direct or indirect interactions with other nuclear factors. My experimental approach illustrates how studies of exogenous receptors in oocytes, in conjunction with microinjected auxiliary proteins, may contribute to the characterization of cell-specific and TRE-mediated mechanisms that modulate the action of receptor domains.

III.

The v-*erbA* Oncogene Initiates Early and Intermediate Events of Meiotic Maturation in *Xenopus* Oocytes

1. Introduction

This chapter presents evidence to suggest that a direct link may exist between partial growth factor autonomy conferred by v-ErbA and induction of gene(s) normally controlled by mitogenic signalling networks. I show that v-ErbA can initiate the release from G_2 cell cycle arrest in stage VI (Dumont, 1972) *Xenopus* oocytes by activating gene expression. Ultrastructural analysis of v-*erbA*-expressing oocytes revealed transformations of the nuclear membrane indicative of early and intermediate stages of meiosis, while chromosome condensation and spindle formation did not occur. A portion of v-ErbA was localized to the cytoplasmic fibrils of the nuclear pore complexes, which raises the possibility that v-ErbA might affect the nucleocytoplasmic transport of a cell cycle regulator.

At the beginning of the introduction, the present model of growth-promotion by v-ErbA is reviewed. The following sections describe the regulation of meiotic pathways by progesterone or by introduced oncogenic proteins, and the

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Abbreviations used in this chapter : AL, annulate lamellae; AP-1, activator protein-1; GVM, germinal vesicle migration; GVBD, germinal vesicle breakdown; IBMX, isobutylmethylxanthine; MPF, maturation-promoting factor; MAPK, mitogen-activated protein kinase; NPC, nuclear pore complex; NLS, nuclear localization signal; RAR, retinoic acid receptor; TR; thyroid hormone receptor.

accompanying structural changes in the nucleus and the nuclear membrane. In addition, the structure of nuclear pore complexes and their function in nucleocytoplasmic transport is illustrated.

(1) *Deregulation of proliferative controls by v-ErbA*

Neoplastic transformation is mediated by the disruption of the signalling networks which regulate cellular proliferation and differentiation. Extensive interference with cellular growth control is exemplified by the cooperative action of v-*erbA* and its retroviral partner v-*erbB*, both carried by the avian erythroblastosis virus (AEV), which together induce acute erythroblastosis and fibrosarcomas in young chicks (Graf and Beug, 1983). The v-*erbB* gene, a truncated version of the chicken epidermal growth factor receptor, is necessary and sufficient for the transformation of chicken embryo fibroblasts by AEV (Sealy *et al.*, 1983; Frykberg *et al.*, 1983). v-*ErbB* initially induces proliferation of erythroid stem cells, but does not block their differentiation program (Frykberg *et al.*, 1983). As a consequence, erythrocytic cells transformed by viruses carrying only the v-*erbB* oncogene spontaneously differentiate at a high rate and stop multiplying (Frykberg *et al.*, 1983; Yamamoto *et al.*, 1983). v-*erbA*, acting in conjunction with v-*erbB*, is able to block differentiation and to inhibit the expression of genes required for the mature phenotype (Fuerstenberg *et al.*, 1992; Schroeder *et al.*, 1990; reviewed in Privalsky, 1992; Damm, 1993). v-ErbA arrests the stem cells transformed by v-ErbB at the CFU-E (colony-forming unit-erythroid) stage (Samarut and Gazzolo, 1982; Beug *et al.*, 1982) and thereby maintains their proliferative potential (Frykberg *et al.*, 1983). Other oncogenes encoding cytoplasmic proteins, like v-*src*, v-*fps*, v-*sea*, and v-Ha-*ras* are able to

replace v-*erbB* by cooperating with v-*erbA* in erythroid transformation (Kahn *et al.*, 1986).

As described in detail in Chapter II, the v-*erbA* product dominantly represses the actions of normal TR and RAR. While chicken embryo fibroblasts expressing only v-*erbA* do not display a fully transformed phenotype, they exhibit a greatly enhanced growth potential and a decreased requirement for growth factors (Gandrillon *et al.*, 1987). These growth-promoting properties of v-ErbA have so far exclusively been linked to dominant repression of the anti-mitogenic roles of TR and RAR. This mode of action is indicated by the fact that v-*erbA* is able to stimulate proliferation by overcoming growth inhibition by retinoic acid (Sharif and Privalsky, 1991). In this context, v-*erbA* function is strongly correlated with its action as a dominant negative oncogene in abrogating AP-1 repression by RAR and TR (Desbois *et al.*, 1991a, 1991b; Zhang *et al.*, 1991). Furthermore, v-erbA renders erythrocytic progenitor cells insensitive to the induction of apoptosis, and of self-renewal inhibition, by retinoic acid and thyroid hormone (Gandrillon *et al.*, 1994).

(2) *Meiotic induction of Xenopus laevis oocytes by progesterone and oncogene products*

Oncogene involvement in the signal transduction events of the cell cycle can be studied by maturation assays carried out in oocytes from *Xenopus laevis*. Fully grown oocytes are arrested at the G_2/M border and can be induced to enter M-phase by progesterone, insulin, or a range of introduced oncogene products (reviewed in Smith, 1989; Daar *et al.*, 1991; Graziani *et al.*, 1992; Grieco *et al.*, 1995). Within minutes of progesterone exposure, a decrease in levels of cyclic AMP (cAMP)

occurs, mediated by inhibition of adenylate cyclase, which in turn downregulates protein kinase A (PKA) and protein kinase C (PKC) activities (Smith, 1989; Matten *et al.*, 1994). Following hormonal stimulation, *de novo* translation of c-*mos* kinase occurs (Sagata *et al.*, 1988; Yew *et al.*, 1992; Sheets *et al.*, 1995) and a number of other cytoplamic kinases are activated, including Raf-1 serine-threonine kinase (Fabian *et al.*, 1993; Muslin *et al.*, 1993b), phosphatidylinositol 3-kinase (Muslin *et al.*, 1993a) and MAPK kinase (Mordret, 1993; Posada *et al.*, 1993; Kosako *et al.*, 1994a, 1994b). These phosphorylation cascades culminate in the activation of maturation-promoting factor (MPF) and mitogen-activated protein kinase (MAPK). MPF and MAPK trigger the events of maturation, including germinal vesicle breakdown (GVBD), chromosome condensation, and meiotic spindle formation, either directly or by activating other protein kinases (Bement and Capco, 1990; Smith, 1989). MPF contains the *Xenopus* homolog of the universal M-phase inducer $p34^{cdc2}$, a serine-threonine kinase with histone H1 phosphorylating activity, which is complexed with B-type cyclins (Gerhart *et al.*, 1984; Maller, 1990; Minshull *et al.*, 1991).

There is growing evidence for the existence of several complementary control pathways which regulate partially overlapping sets of events at the G_2/M transition. In particular, early maturation events, occurring prior to chromosome condensation, can be induced in progesterone-treated oocytes in the absence of protein synthesis (Baltus *et al.*, 1973; Drury and Schorderet-Slatkine, 1975); membrane vesicularization, lamin disassembly and chromosome condensation are independent processes (Newport and Spann, 1987; Bement and Capco, 1990); GVBD can occur in the absence of active CDC2 in mouse oocytes (Choi *et al.*, 1991; Gavin *et al.*, 1992,

1994); and GVBD and white spot formation in *Xenopus* oocytes can be biochemically separated (Muramatsu *et al.*, 1989; Waldmann *et al.*, 1990). In addition, GVBD in *Xenopus* oocytes and a pseudomitotic phenotype in HeLa cells are inducible by a *cdc2*-independent, NIMA-like pathway (Osmani *et al.*, 1991a, 1991b; Lu and Hunter, 1995).

(3) *Nuclear envelope dynamics during the cell cycle*

The nuclear membrane of eukaryotic cells separates the genome from the cytoplasm. This separation permits additional levels of regulation which are absent in prokaryotic cells, and thus constitutes a major landmark in evolution (Dingwall and Laskey, 1992). The nuclear membrane consists of two concentric lipid bilayers, which are perforated by nuclear pore complexes (NPCs) that serve as channels for molecular exchanges between the nucleus and cytoplasm. The inner nuclear membrane is lined by the nuclear lamina, composed of a network of lamin proteins, a specialized type of intermediate filament protein (reviewed in Dingwall and Laskey, 1992). At the beginning of M-phase in almost all eukaryotic cells, the nuclear DNA dissociates from the nuclear envelope and condenses to form compact chromosomes. In eukaryotic cells that undergo an open mitosis, the nuclear envelope vesicularizes and the nuclear lamins depolymerize. At the completion of mitosis, these membrane vesicles and lamin proteins bind to the surface of condensed DNA and reestablish an intact nuclear envelope (reviewed in Gerace and Foisner, 1994; Goldberg and Allen, 1995; Dingwall and Laskey, 1992). Of all the mitotic rearrangements that occur, the disassembly of the nuclear lamina is the best understood, whereas almost nothing is known about the mechanisms of membrane vesicularization and nuclear pore

disassembly.

The *Xenopus* oocyte nuclear membrane is an attractive model system for the investigation of dynamic interactions of its components during the passage through the cell cycle (reviewed in Gerace and Foisner, 1994). The nuclear membrane of the *Xenopus* oocyte possesses several specialized features, i.e., a high density of NPCs (Scheer *et al.*, 1976), and a nuclear lamina composed of a single lamin isomer (Benavente *et al.*, 1985; Stick and Hausen, 1985); moreover, the large size of the oocyte nuclei allows the manual isolation of nucler membranes (Krohne and Franke, 1983). Cell-free systems, based on extracts from mature *Xenopus* eggs in either a mitotic or an interphase state, have allowed the elucidation of a number of processes involved in nuclear disassembly (see, for example, Lohka and Maller, 1985; Macaulay *et al.*, 1995; Miake-Lye and Kirschner, 1985; Newport and Spann, 1987; Pfaller *et al.*, 1991; Pfaller and Newport, 1995; reviewed in Gerace and Foisner, 1994) and nuclear reassembly respectively (see, for example, Lohka and Masui, 1984; Burke and Gerace, 1986; Newport, 1987; Pfaller *et al.*, 1991; Pfaller and Newport, 1995; reviewed in Almouzni and Wolffe, 1993b). Importantly, employing cell-free nuclear disassembly systems, it has been possible to dissociate GVBD from chromosome condensation (Newport and Spann, 1987). Lamina disassembly appears to be necessary but not sufficient for GVBD (Newport and Spann, 1987), and is controlled by reversible phosphorylation of lamin proteins (Miake-Lye and Kirschner, 1985; Burke and Gerace, 1986; Gerace and Blobel, 1980). MPF, the pivotal regulator of mitosis, is thought to play a direct role in the phosphorylation of the lamins at the onset of M-phase (Peter *et al.*, 1990). Finally, nuclear reassembly can be studied in *Xenopus* egg extracts. These extracts, which contain large stockpiles of

disassembled nuclear components, readily reconstitute nuclei around condensed chromosomes *in vitro*; a process which occurs with astonishing spontaneity (Lohka and Masui, 1983; Burke and Gerace, 1986; Newport, 1987).

Annulate lamellae (AL), an additional intracellular membrane compartment, are found in the cytoplasm and consist of stacks of flattened membranes perforated by numerous and densely packed pore complexes (Kessel, 1992). AL are especially numerous in rapidly growing cells, such as male and female gametes, tumour cells and virally infected cells. AL are believed to store excess nuclear envelope and NPC components for later use, but might have additional functional roles (Kessel, 1992). AL disassemble, along with the oocyte nucleus, during meiotic maturation (Stafstrom and Staehelin, 1984; Bement and Capco, 1989; Kessel and Subtelny, 1981). During this process, pore complexes disappear and the membrane is transformed into distended cisternae (Kessel and Subtelny, 1981). The significance of these structural alterations remains unknown.

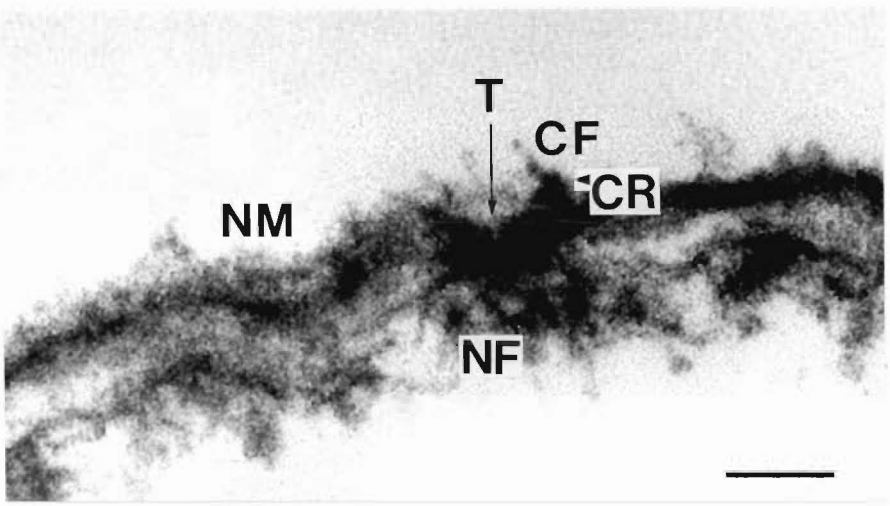
(4) *Structure and function of nuclear pore complexes*

Nucleocytoplasmic transport plays a fundamental role in coordinating the functions of the nucleus and the cytoplasm (reviewed in Melchior and Gerace, 1995; Stochaj and Silver, 1992). Transport across the nuclear envelope is carried out by the NPCs which are believed to be similar in both structure and function in all eukaryotic organisms from yeast to mammals (reviewed in Forbes, 1992; Gerace, 1992; Hurt, 1993; Rout and Wente, 1994). The nuclear pore is a highly selective channel through which macromolecules must pass to enter or exit the nucleus (reviewed in Hinshaw, 1994). Most proteins are too large to diffuse through the NPCs and are

actively transported through gated channels in the NPCs by signal-mediated mechanisms (reviewed in Guiochon-Mantel and Milgrom, 1993; Melchior and Gerace, 1995; Stochaj and Silver, 1992). Mediated import of proteins into the nucleus is specified by short stretches of amino acids known as nuclear localization sequences (NLSs). NLSs do not possess a clear consensus sequence, but most NLSs are highly enriched in basic amino acids, and may be continuous, as in the simian virus 40 (SV40) large T antigen, or bipartite, as in nucleoplasmin (Boulikas, 1994; LaCasse and Lefebvre, 1995; reviewed in Dingwall and Laskey, 1991). NLS-mediated nuclear import of proteins can be viewed as a two-step process, consisting of ATP-independent binding to the NPC, followed by ATP-dependent translocation through the pore channel (Görlich *et al.*, 1994; Newmeyer *et al.*, 1986a, 1986b; Newmeyer and Forbes, 1988; Richardson *et al.*, 1988).

The NPC has dimensions of ~120 x 80 nm and a mass of ~125 x 10^6 D (Reichelt *et al.*, 1990), and may contain up to 100 different polypeptides (Rout and Wente, 1994). Of these pore proteins, only a small number have been identified to date (reviewed in Rout and Wente, 1994; Doye and Hurt, 1995). Three-dimensional image reconstruction suggests that NPCs are comprised of nuclear and cytoplasmic rings, with eight spokes radiating toward a central transporter apparatus (Hinshaw *et al.*, 1992; Hinshaw, 1994) (Fig. 16). A "central channel complex" is attached to the inside of the spokes and contains the gated channel involved in signal-mediated transport of macromolecules (Forbes, 1992; Hinshaw, 1994; Hinshaw *et al.*, 1992; Melchior and Gerace, 1995). In addition, sets of fibrils emanate from both the nucleoplasmic and cytoplasmic rings (Forbes, 1992; Richardson *et al.*, 1988; Ris and Malecki, 1993). In isolated nuclear envelopes of amphibian oocytes, the

Figure 16. Transverse thin section of a nuclear pore complex (NPC) in an isolated nuclear membrane from a stage V (Dumont, 1972) *Xenopus* oocyte. NM, nuclear membrane; CR, cytoplasmic ring; T, central transporter; CF, cytoplasmic fibrils; NF, nucleoplasmic fibrils. Bar: 50 nm.


T
CF
CR
NM
NF

nucleoplasmic fibrils, which are ~75-100 nm long, are joined at their distal ends in a basket-like structure (Jarnik and Aebi, 1991; Goldberg and Allen, 1992), whereas the structurally distinct cytoplasmic fibrils are <25-50 nm long and have no obvious distal connections (Forbes, 1992) (Fig. 16). The initial binding of NLS-bearing proteins, complexed with cytoplasmic transport factors, is thought to occur at the fibrils at the cytoplasmic rings of the NPCs (reviewed in Melchior and Gerace, 1995).

NPCs perform a crucial role in controlling cellular processes, and mutations in NPC proteins can cause nuclear transport defects, extensive changes in nuclear membrane structure, and abnormal nuclear migration in the yeast *Saccharomyces cerevisiae* (see, for example, Bogerd *et al.*, 1994; for a recent review, see Doye and Hurt, 1995). Interestingly, certain viral proteins can modulate nucleocytoplasmic transport of mRNA. For example, the adenoviral E1B protein, bearing a NLS and localized at NPCs, inhibits export of host transcripts, while promoting export of viral mRNA (Liang *et al.*, 1995). Other recent studies showed that the fibrils emanating from the cytoplasmic face of NPCs contain the proteins CAN/nup214 (Fornerod *et al.*, 1995; Kraemer *et al.*, 1994) and TPR/nup265 (Byrd *et al.*, 1994), which have both been associated with oncogenesis in humans when fused to other genes. Thus, oncogenes may be capable of inducing oncogenesis by altering the functions of NPCs.

(5) *Summary of results*

To assess whether v-ErbA can interfere directly with cell cycle control pathways, I investigated the effects of expression of v-*erbA* in *Xenopus* oocytes. This study showed that v-ErbA initiates early to intermediate meiotic events in *Xenopus* oocytes. v-ErbA induced a rearrangement of pigment in the animal hemisphere resembling the meiotic white spot and extensive ultrastructural meiotic changes in the architecture of the nuclear membrane, annulate lamellae, and the nucleoli, while chromosome condensation and spindle formation did not occur. Meiotic induction by v-ErbA was not mimicked by a dominant negative *in vitro*-generated mutant of TR, suggesting that release from G_2 cell cycle arrest by v-ErbA was not mediated by dominant repression of endogenous TR. Furthermore, v-ErbA-initiated maturation was independent of the cAMP/MPF signal pathway, but required *de novo* gene expression. Interestingly, the v-*erbA* protein was partially localized to NPCs by immunogold labelling. A novel function is proposed for v-ErbA by which it might disrupt nuclear mitogenic controls.

2. Materials and Methods

(1) *Plasmids*

RS-v-erbA, RSh-TRß C122>A and pRSV-*lacZ* are described in Chapter II, Materials and Methods. The pKCR2-cea plasmid was a gift of B. Vennström (Karolinska Institute, Stockholm, Sweden), and contains the chicken *c-erbA* cDNA expressed from the SV40 promoter (Sap *et al.*, 1989). The pGEM-4Z vector was a gift of M. Privalsky (University of California, Davis, CA), and contains the *gag*-v-*erbA* cDNA under control of the SP6 promoter. The V_3 clone was a gift of A. Munoz (Instituto de Investigaciones Biomedicas, Madrid, Spain). The chimeric v-/c-*erbA* clone V_3 contains the retroviral *gag* sequence in the *Sac*I-*Dra*III fragment of v-*erbA* joined to the subsequent *Dra*III-*Apa*I fragment of c-*erbA* under control of the phage T7 promoter (Munoz *et al.*, 1988).

(2) *Oocyte microinjections*

A lobe of ovary was surgically removed from an adult female *Xenopus laevis* and processed as described in Chapter II. Microinjections were performed according to published methods with modifications (see Chapter II). Defolliculated stage VI (Dumont, 1972) *Xenopus laevis* oocytes were microinjected with 5 ng v-*erbA* expression vector alone or with 2 ng pRSV-*lacZ*, or with 5 ng pRSV-*lacZ* as a control, in a fixed volume of 20 nl TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) into the nucleus by the 'blind' injection method with the needle inserted in the center of the animal pole. Alternatively, 5 ng V_3 mRNA in a fixed volume of 50 nl TE, pH 7.6, was injected into the cytoplasm. In addition, internally labelled v-*erbA* protein

was synthesized by Caroline F. Bunn from the pGEM-4Z template by *in vitro* coupled transcription/translation in rabbit reticulocyte lysate (Promega, Madison, WI) according to the manufacturer's specifications, using SP6 polymerase. Oocytes were incubated in cycloheximide (200 µg/ml) or actinomycin D (30 µg/ml) for 16 h, and subsequently microinjected into the cytoplasm with 50 nl v-*erbA* solution (approximately 100 pg v-ErbA in rabbit reticulocyte lysate). This procedure was also carried out by C. F. Bunn.

(3) *ELISA*

Thirty-six hours after microinjection of 2 ng pRSV-*lacZ* and 5 ng RS-v-erbA, healthy oocytes (uniform pigment, not mottled) were homogenized singly in 200 µl 0.25 M Tris buffer, pH 7.5, and centrifuged at 10,000 x *g* in a microfuge at 4°C for 10 min. The supernatant was then centrifuged as before. This extract was used in the determination of ß-galactosidase protein expression levels by enzyme linked immunosorbant assay (ELISA) according to the manufacturer's specifications (5 Prime-3 Prime, Boulder, CO), and as described in Chapter II. This assay was repeated two times with oocytes from different animals since there can be variability in transcriptional activity between different batches of oocytes (see Chapter II). For each assay, a standard curve utilizing four pure protein standards was prepared, to ensure that ß-Gal concentrations of sample extracts fell within the linear range of the assay. The data on reporter gene expression levels were analyzed by nested analysis of variance (ANOVA) controlling for variability between females and between independent ELISA assays.

(4) *Incubation treatments and assessment of germinal vesicle movement and germinal vesicle breakdown*

Oocytes were incubated in (i) O-R2 medium (Allison *et al.* 1991) only; or (ii) progesterone (10 μg/ml) (Sigma, Chemical Co., St Louis, MO); (iii) taxol (10 μg/ml) (Sigma); (iv) forskolin (20 μM) (Sigma) and isobutylmethylxanthine (IBMX; 2 mM) (Sigma); (v) cycloheximide (200 μg/ml) (BDH Chemicals NZ Ltd., Palmerston North, New Zealand); or (vi) actinomycin D (30 μg/ml) (actinomycin CI; Boehringer Mannheim NZ Ltd., Auckland, New Zealand) in O-R2. Twenty-four hours after microinjection with 5 ng RS-v-erbA, pRSV-*lacZ*, or 50 nl v-*erbA* protein solution, and/or a 24-hour incubation treatment ([i]-[vi]), oocytes were scored for germinal vesicle movement (GVM, white spot) under a stereomicroscope. Oocytes were assessed for germinal vesicle breakdown (GVBD) by manual dissection after fixation in ice-cold 1% trichloroacetic acid (TCA) for 5 min. Oocytes were scored positive for GVBD when the nucleus could not be dissected intact due to nuclear instability.

(5) *Differential interference contrast microscopy*

Oocytes, incubated in progesterone, or microinjected with 5 ng RS-v-erbA, RSh-TRß C122>A, or unrelated RSV-*lacZ* template, were fixed and embedded essentially as described by Hausen and Riebesell (1991). In brief, oocytes were fixed in Romeis fixative (25 ml saturated mercuric chloride, 20 ml 5% trichloroacetic acid, 15 ml 37% formaldehyde) for three hours. Subsequently, oocytes were dehydrated in an ethanol series of 50% (15 min), 70% (30 min), 80% (15 min), 90% (15 min), 95% (15 min), and 100% (3 x 10 min); then infiltrated in 50% glycol methacrylate infiltration solution (Polaron) in ethanol for 1 hour and left in 100% infiltration

solution overnight. Oocytes were embedded in glycol methacrylate (Polaron) in gelatine capsules under nitrogen at 37°C for 5 hours. Five micron sections were obtained with Ralph glass knives. In order to remove mercury precipitates, the dry sections were treated with an alcoholic iodine potassium iodide solution (2% iodine, 3% potassium iodide in 90% ethanol) for 2 min, washed in 0.25% sodium thiosulfate for 15 min, and thoroughly rinsed in distilled water. The sections were stained utilizing an azofuchsin/aniline blue/orange G triple staining method (Hausen and Riebesell, 1991). Three independent batches of oocytes were used, and 3 to 4 oocytes per treatment/per batch were analyzed using differential interference contrast microscopy. Oocyte sections were photographed using Agfa optima color film with a blue filter.

(6) *Electron microscopy for ultrastructural analysis*

In ultrastructural studies of *Xenopus* oocytes, long fixation times and special fixation procedures are necessary. Unless indicated otherwise, all procedures were carried out at 4°C. Oocytes were fixed in 2.5% glutaraldehyde and 0.05% low molecular weight tannic acid in O-R2 overnight. Oocytes were then washed in O-R2 three times for 30 min, and subsequently transferred to 2% OsO_4 (in ddH_2O) for 3 hours with one change. Oocytes were then washed in O-R2 for 30 min, and dehydrated in a graded ethanol series of 30% (30 min), 50% (30 min), 70% (overnight), 80% (20 min), 90% (20 min), 95% (15 min), and 100% (2 x 15 min). After the 70% ethanol step, all further procedures were carried out at room temperature. Oocytes were incubated in 100% acetone twice for 20 min, and then gradually embedded in Spurr's resin in acetone: 30% resin (1 hour), 50% resin

(3 hours), 70% resin (overnight), 90% resin (8 hours), and 100% resin (overnight). Oocytes were transferred into 100% resin in molds and cured at 70°C for 24 hours. Ultra-thin sections (50 nm) were stained with 5% uranyl acetate in 50% ethanol for 20 min, washed in ddH_2O, stained with lead citrate for 20 min, and put through a final washing step in ddH_2O.

(7) ***Histone H1 kinase assay***

Adult female *Xenopus laevis* were injected with 0.5 ml gonadotropin (from pregnant mares' serum, Sigma; 200 U/ml in sterile ddH_2O) subcutaneously into the dorsal lymph sac, to improve the synchrony of response to progesterone in oocytes. After four days, a lobe of ovary was surgically removed from the animal and processed as described above. Defolliculated stage VI (Dumont, 1972) oocytes were microinjected into the nucleus with 5 ng RS-v-erbA or incubated in progesterone (10 µg/ml). At specified times, oocytes extracts were prepared by homogenizing groups of 10 oocytes in 20 µl extraction buffer (20 mM Tris, pH 7.5, 80 mM ß-glycerophosphate, 20 mM EGTA, 15 mM $MgCl_2$, 25 µg/ml aprotinin, 25 µg/ml leupeptin, 1 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride [PMSF], 5 mM Pefabloc [Boehringer Mannheim], and 1 mM dithiothreitol [DTT]). The homogenate was centrifuged at 10,000 *x g* for 10 min at 4°C to pellet yolk and pigment. Ten microliters of the supernatant were mixed and incubated for 20 min at 25°C with 10 µl kinase buffer (30 mM Tris, pH 7.5, 30 mM $MgCl_2$, 1 mCi/ml $\gamma[^{32}P]$ ATP [Amersham], 3 mg/ml histone H1[Sigma]) (Kosako *et al.*, 1994b). The reaction was stopped by the addition of an equal volume of 2 X SDS sample buffer (4% SDS, 20% glycerol, 120 mM Tris, pH 6.8, 0.01% bromophenol blue) and boiling for 5

min. Samples were resolved by 12% SDS-polyacrylamide electrophoresis followed by autoradiography.

(8) *In vitro transcription of the V_3 construct*

The plasmid containing the chimeric *v-/c-erbA* gene V_3 under the control of the phage T7 promoter was linearized with *Eco* RI, extracted with phenol-chloroform and precipitated. Transcription was carried out with 1 µl T7 RNA polymerase (50 U/µl, Epicentre) in 4 µl 5X T7 RNA polymerase buffer (Epicentre), 2 µl DDT (100 mM), 1 µl RNasin (40 U/µl) (Promega), 0.6 µl of each of the four ribonucleotide triphosphates (100 mM), 3 µl ^{7}mGpppG (20 mM) (New England Biolabs), and 1 µl template DNA at a concentration of 1 µg/µl, for 1h at 37°C. The mixture was then incubated with 1 µl of RNase-free DNase (10 U/µl) (Boehringer) in 9 µl DNase dilution buffer for 15 min at 37°C. The reaction was stopped by heating the mixture to 65°C, and the mRNA was precipitated with 2 µl 4 M LiCl and 60 µl of 100% ethanol, prechilled at -20°C, for 30 min at -80°C. The RNA was pelleted by centrifugation at 12,000 *x g* for 10 min, washed with cold 70% ethanol, dried under vacuum, and dissolved in 5 µl TE, pH 7.6.

(9) *Immunoprecipitation of oocyte fractions with an anti-v-erbA antibody*

Oocytes were microinjected with 5 ng RS-v-erbA, pKCR2-cea expression plasmid into the nucleus, or 5 ng V_3 mRNA into the cytoplasm, and incubated in a sterile microtitre plate (5 oocytes/well) in 30 µl of O-R2 with 1 mCi/ml L-[^{35}S] methionine (1000 Ci/mmol, Amersham Pty Ltd., Auckland, New Zealand) at 18°C for 24 h. Nuclear fractions of 10 or 20 pooled v-*erbA*-injected oocytes were prepared manually

as described in Chapter II, after fixation in 1% ice-cold TCA for 5 min. Samples were homogenized in 0.5 ml NET-2 (Allison et al., 1993) containing 0.1 mM PMSF. For preparation of nuclear membrane fractions, nuclei were manually isolated in nuclear isolation medium (NIM; 83 mM KCl, 17 mM NaCl, 10 mM Tris, pH 7.2, 5 mM Pefabloc) (Krohne and Franke, 1983) in a small siliconized petri dish, using watchmaker's forceps. Nuclei were freed from adhering yolk by aspirating them into a pipette with an inner bore diameter of 0.7-0.8 mm, then washed once in fresh NIM and transferred into nuclear envelope isolation medium (NEM; 83 mM KCl, 17 mM NaCl, 10 mM $MgCl_2$, 10 mM Tris, pH 7.2, 5 mM Pefabloc). Within 30-60 s, the nuclear content forms an opaque and compact aggregate from which the nuclear membrane (envelope) detaches. The nuclear membranes were isolated by aspirating nuclei into a pipette with an inner bore diameter of 0.3-0.4 mm, cleaned in fresh NEM by repeated aspiration into a small pipette and transferred into a microfuge tube. Ten or 20 nuclear envelopes were pooled per sample and collected by centrifugation for 4 min at 8,000 *x g* and resuspended by gentle aspiration in 0.5 ml NET-2 containing 5 mM Pefabloc.

For total soluble protein electrophoretic separations, 20 µl 2 X SDS sample buffer and 1 mM DTT were added to 20 µl homogenate from nuclear and nuclear membrane fractions, which were then resolved by 12% SDS-polyacrylamide gel electrophoresis and stained with Bio-Rad Silver Stain (Bio-Rad Laboratories Pty Ltd., Auckland, New Zealand). Immunoprecipitations of the nuclear fraction and nuclear envelope samples were carried out as described in Chapter II, using 30 µl of monoclonal antibody LA038 against v-*erbA* residues 58-75.

(10) ***Immunolabelling of isolated nuclear membranes***

Oocytes were microinjected with 5 ng RS-v-erbA or pKCR2-cea into the nucleus and incubated in O-R2 for 12 to 14 h. Nuclear membranes of 20 oocytes per sample were manually isolated as described above, and fixed in 2% formaldehyde, freshly made from paraformaldehyde in PBS (137 mM NaCl, 1.3 mM Na_2HPO_4, 2.5 mM NH_2PO_4, pH 7.2), for 10 min at 4°C. Immunolabelling with colloidal gold, employing a biotin-streptavidin bridging technique, was carried out following published procedures (Cordes *et al.*, 1993) with modifications, and according to the manufacturer's specifications (Amersham). Membranes were collected by centrifugation at 8,000 *x g* for 4 min and washed three times in PBS, pH 7.2, containing 50 mM NH_4Cl. Membrane samples were then resuspended in 24 µl PBS, pH 7.2, with 6 µl anti-v-*erbA* antibody LA038, and incubated for 2 h at room temp. Membranes were then centrifuged at 8,000 *x g* for 4 min, and washed three times in PBS. Subsequently, membranes were resuspended in 99 µl PBS, pH 7.2, containing 1 µl biotinylated goat anti-mouse Ig antibody (Amersham), and incubated for 2 h at room temperature. The samples were then washed with PBS, pH 7.2, as described above. Next, membranes were incubated in 38 µl PBS, pH 7.2, with 2 µl AuroProbe EM streptavidin 10 nm (Amersham) for 2 h at room temperature, and washed in PBS as before. Membranes were fixed in 2.5% glutaraldehyde in PBS, pH 7.2, for 30 min at 4°C, washed three times in PBS, followed by fixation in 2% aqueous OsO_4 for 30 min at 4°C and several washes in ddH_2O. Membrane samples were dehydrated in a graded ethanol series, incubated in 100% acetone twice for 20 min, and embedded in Spurr's resin in acetone: 50% resin (1 h), 90% resin (overnight), and 100% resin (1 h). Membranes were then immersed in fresh resin and cured at 70°C for 14 h.

Ultra-thin sections (50 nm) were stained with 2% uranyl acetate in 50% ethanol for 10 min, washed in ddH_2O, stained with lead citrate for 5 min, and washed in ddH_2O.

3. Results

(1) *v-ErbA induced GVM and GVBD in Xenopus oocytes, but did not activate histone H1 kinase activity*

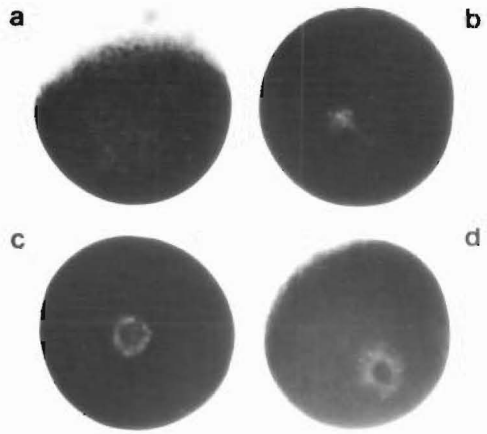
During meiosis, oocytes undergo unequal divisions leading to polar body formation. For this process to occur, the oocyte nucleus (germinal vesicle) situated near the center of the oocyte, must migrate towards the animal hemisphere cortex and pigment granules in the region are rearranged, producing a whitish circular spot (germinal vesicle migration, GVM) (Lessman, 1987; reviewed in Smith, 1989). At the end of progesterone-induced GVM and GVBD, the white spot represents the area where the meiotic spindle has formed (Brachet *et al.*, 1970; reviewed in Smith, 1989; Bement and Capco, 1990). Thus, the meiotically mature egg can be distinguished from the oocyte by the formation of this white spot which becomes delineated by a dark ring of rearranged pigment.

I first tested the ability of v-ErbA to initiate maturation in *Xenopus* oocytes by comparing the rate of GVM and GVBD in progesterone-treated and v-*erbA*-injected oocytes obtained from the same females. I have shown previously that v-ErbA, synthesized from a microinjected gene template in *Xenopus* oocytes, is functional and acts as a dominant repressor of TR (Chapter II; Nagl *et al.*, 1995). In the present series of experiments, stage VI oocytes (Dumont, 1972) were injected into the

nucleus with 5 ng of v-*erbA* expression plasmid and cultured in O-R2 for 24 h, or incubated in progesterone for the same time period. Microinjection of v-*erbA* expression plasmid resulted in GVM. After 24 h, pigment granules were rearranged at the animal hemisphere cortex, forming a distinct, translucent circular spot with the nucleus visible underneath (Fig. 17A). The v-ErbA-induced pigment rearrangement was highly similar to the meiotic white spot induced by progesterone (Fig. 17A, cf. *Panels b* and *c*), although occasionally pigment aggregation in the center of the white spot in v-*erbA*-injected oocytes was seen (*Panel d*). For an assessment of whether GVM in v-*erbA*-injected oocytes is an active process or a consequence of degenerative changes in the cytoskeleton, I compared GVM in v-*erbA*-expressing oocytes with acrylamide-induced GVM (Lessman and Kessel, 1992). At present, the mechanism for positioning the oocyte nucleus is unknown, but is believed to involve an extensive intermediate filament network (Lessman and Kessel, 1992). Acrylamide, an intermediate filament inhibitor, induced GVM in 20.5% of control oocytes positioned with the animal pole uppermost, but only elicited GVM in 2.6% of controls positioned in the opposite orientation (Table I). In contrast, 56.8% of v-*erbA*-injected oocytes exhibited a distinct white spot when orientated with the animal pole uppermost, while 27.8% of v-*erbA*-injected oocytes showed this feature when incubated with the vegetal pole uppermost. The depigmented patch at the animal pole of oocytes incubated with acrylamide, resulting from the displacement of pigment granules by the nucleus (data not shown), was distinct from the white spot seen in v-*erbA*-expressing oocytes (Fig. 17A). GVM occurred in 41% of v-*erbA*-injected oocytes oriented at random, while the mean frequency of GVM in

Figure 17. v-ErbA induces GVM and GVBD in *Xenopus* oocytes, but not MPF histone H1 kinase activity or chromosome condensation. (*A*) v-ErbA induced pigment rearrangements similar to meiotic white spot formation. Stage VI (Dumont, 1972) oocytes were injected with 5 ng *lacZ* expression plasmid (*Panel a*), incubated in 10 μg/ml progesterone (*Panel b*), or injected with 5 ng v-*erbA* template (*Panels c* and *d*), followed by examination of the pigment arrangement after 24 h. (*B*) GVM and GVBD induced by v-ErbA were not inhibited by forskolin/IBMX or taxol. Stage VI oocytes were injected with 5 ng v-*erbA* template (V) or 5 ng *lacZ* expression vector (C) and incubated in O-R2 alone, or in 10 μg/ml progesterone (P). Alternatively, microinjected or progesterone-treated oocytes were incubated in 20 μM forskolin/2 mM IBMX (forskolin), or 10 μg/ml taxol. After 24 h, the percentage of oocytes displaying GVM was assessed by inspection of whole oocytes, and the frequency of GVBD induction was scored by manual dissection of nuclei in 1% TCA. Three independent experiments were performed (30-40 oocytes/treatment/experiment). The error bars indicate the SEMs. (*C*) Chromatin remained decondensed, as v-ErbA did not inhibit transcription of a *lacZ* reporter gene. Oocytes were microinjected with 2 ng *lacZ* reporter gene construct alone (reporter only), or together with 5 ng v-*erbA* template (plus v-ErbA), and incubated for 36 h. Relative *lacZ* gene transcription was expressed in arbitrary units based on measurements of ß-galactosidase protein levels by ELISA. Two independent experiments with 5 oocytes per treatment were performed. The error bars indicate the SEMs ($p < 0.39$). (*D*) v-ErbA did not induce MPF histone H1 kinase activity. After microinjection of 5 ng v-*erbA* or pRSV-*lacZ* template as a control (C) (*upper panel*), or incubation in progesterone or O-R2 (C) (*lower panel*), oocytes were collected at the indicated times and MPF kinase activity was assayed using histone H1 as a substrate and $\gamma[^{32}P]$ ATP. Samples were resolved by 12% SDS-polyacrylamide electrophoresis followed by autoradiography. MPF histone H1 kinase activity was assayed in oocytes with (+) or without (-) a distinct white spot (GVM). A representative example of three independent experiments, using oocytes from differnt female *Xenopus*, is shown.

A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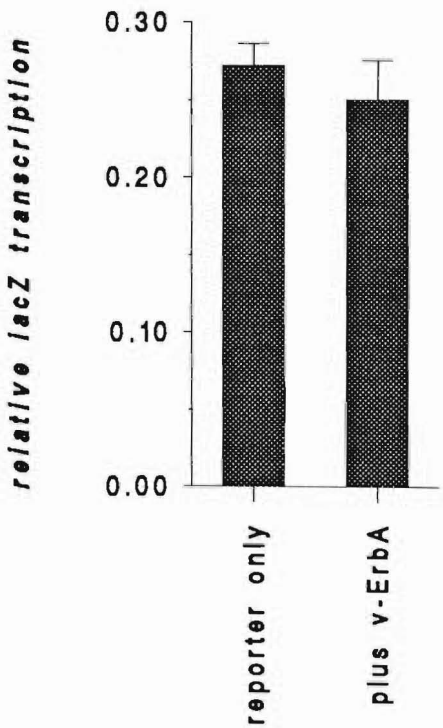
C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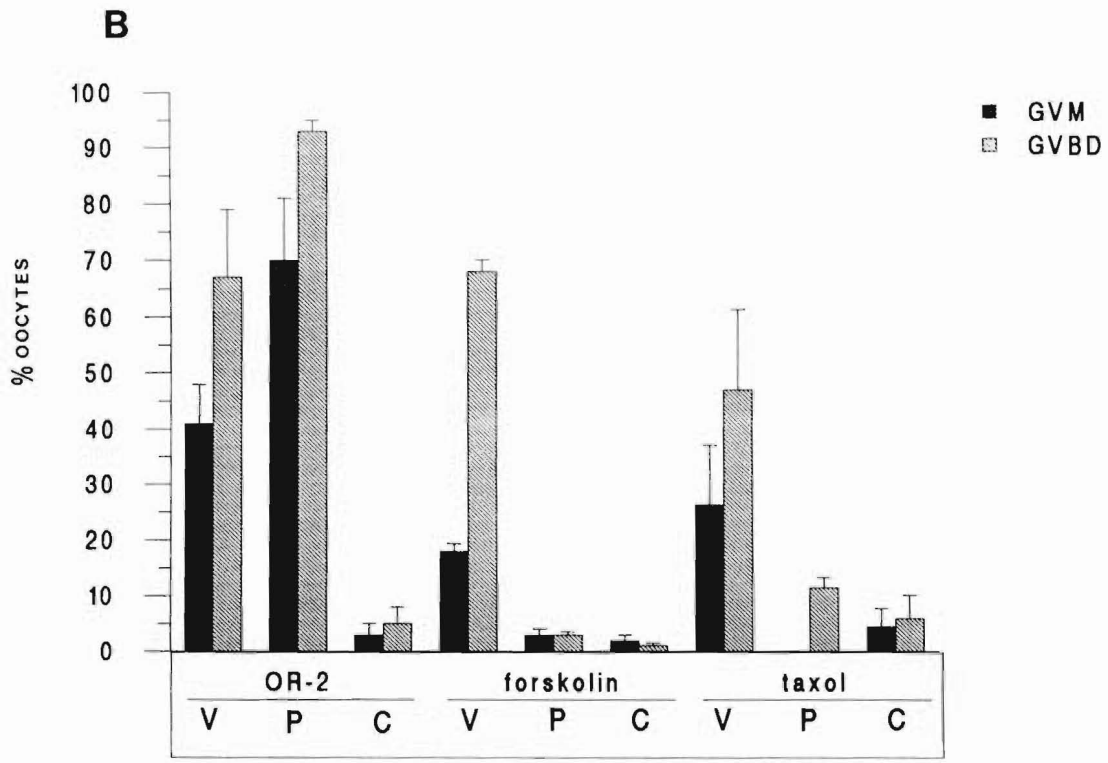
plus v-ErbA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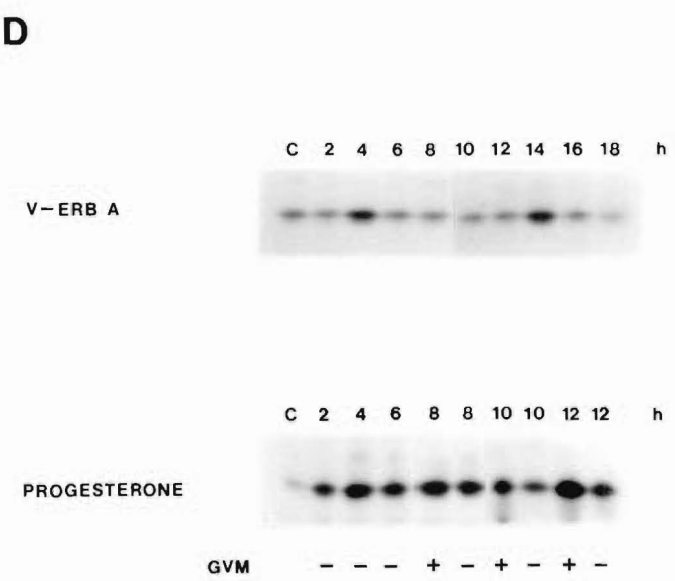

D
C 2 4 6 8 10 12 14 16 18 h
V-ERB A
C 2 4 6 8 8 10 10 12 12 h
PROGESTERONE
GVM
- - - + - + - + -

Table I. GVM in stage VI (Dumont, 1972) *Xenopus laevis* oocytes in the presence of v-ErbA or acrylamide*.

pole uppermost	*animal*			*vegetal*		
		GVM			GVM	
	total # oocytes	#	%	total # oocytes	#	%
v-ErbA	44	25	56.8	43	12	27.8
acrylamide	34	7	20.5	38	1	2.6

* Oocytes were microinjected with 5 ng v-*erbA* expression template or incubated in acrylamide (10 mM) and cultured for 24 h in the orientation indicated, before scoring for GVM.

progesterone-stimulated oocytes was 69% (Fig. 17B). Thus, although positioning the animal pole uppermost facilitated nuclear migration in v-*erbA*-expressing oocytes, GVM was observed with both orientations to gravity, indicating that v-*erbA*-induced GVM is a directed process.

Next, oocytes were assessed for GVBD, and were scored positive when the nucleus could not be dissected intact. In 67% of oocytes that had been injected with v-*erbA* template, nuclei were unstable and could not be dissected intact 24 h after microinjection (Fig. 17B). In progesterone-treated oocytes, GVBD occurred with a mean frequency of 94% after 24 h (Fig. 17B). It has been noted previously that GVM or white spot formation are not always apparent during maturation (Smith, 1989; Bement and Capco, 1990). In the present study, GVBD could be observed in the absence of GVM both in progesterone-treated and v-*erbA*-injected oocytes. Taken together, these findings suggested that expression of v-*erbA* in oocytes caused nuclear changes, which strongly resembled the events associated with maturation.

Substantial evidence exists indicating that the decrease in cAMP which follows progesterone treatment is both necessary and sufficient for meiotic maturation. For example, treatment of oocytes with forskolin, an adenylate cyclase activator, or IBMX, an inhibitor of cAMP breakdown, completely inhibits meiotic maturation (reviewed in Smith, 1989; Bement and Capco, 1990). To test whether v-ErbA activated the cAMP-regulated CDC2/MPF pathway, I incubated v-*erbA*-injected oocytes in forskolin and IBMX. Forskolin and IBMX did not significantly affect GVBD in v-*erbA*-injected oocytes and decreased GVM only moderately, while completely inhibiting progesterone-induced GVM and GVBD (Fig. 17B). This result indicates that the nuclear changes induced by v-ErbA do not require inhibition of

adenylate cyclase and a decrease in cAMP levels.

During meiosis, the organization of microtubules undergoes dramatic changes and eventually tubulin is assembled into the spindle complex. Activated MPF and MAPK alter microtubule dynamics by inducing the phosphorylation of microtubule-associated proteins (MAPs) which causes a drastic decrease in microtubule length (Bement and Capco, 1990; Davis, 1993; Gotoh *et al.*, 1991; Matsuda *et al.*, 1992; Mordret, 1993). Formation of the meiotic spindle, as well as GVM, can be blocked by experimentally interfering with the cytoskeletal targets of MAPKs (reviewed in Davis, 1993; Jessus *et al.*, 1987; Lessman, 1987). Taxol, a microtubule stabilizer, inhibited GVM and GVBD induced by progesterone, but had no significant effect on GVM and GVBD in v-*erbA*-injected oocytes (Fig. 17B). This suggests that the nuclear changes mediated by v-ErbA did not involve rearrangements of the microtubular network; however, this does not rule out a role for other components of the cytoskeleton, such as intermediate filaments (Lessman and Kessel, 1992). Another characteristic of progesterone-induced maturation is chromosome condensation and the cessation of transcriptional activity (Bement and Capco, 1990). Transcription of an unrelated *lacZ* gene template was unaffected by the presence of v-ErbA 36 h after microinjection (Fig. 17C), indicating that gene transcription was not shut down and chromatin remained decondensed.

MPF kinase activation, the definitive parameter of MPF-mediated oocyte maturation, can be measured *in vitro* by means of kinase activity of oocyte extracts, using histone H1 as a substrate (Langan *et al.*, 1989). I assayed H1 histone kinase activity in v-*erbA*-injected and progesterone-incubated oocytes in three independent experiments with oocytes obtained from different female *Xenopus*. The results of a

representative experiment, using oocytes from the same female, are shown in Fig. 17D. In extracts prepared from v-*erbA*-microinjected oocytes, H1 kinase was not activated over an 18 h period when compared with an extract from *lacZ*-injected controls assayed after 2 h (Fig. 17D). The slightly elevated levels of H1 histone kinase activity in samples assayed 4 h and 14 h after microinjection were interpreted as not significant as they were not reproduced in repeat assays. Moreover, similar fluctuations in H1 histone kinase activity were also observed in extracts of *lacZ*-injected controls assayed over time (data not shown). Compared with a control extract from oocytes cultured in O-R2, extracts from oocytes incubated in progesterone exhibited high levels of histone H1 kinase activity after 2 h (2 h, GVM -), approximately 6 h prior to the appearance of the white spot (8 h, GVM +) (Fig. 17D). Both in the presence and absence of GVM, strong H1 histone kinase activity was present in extracts from progesterone-incubated oocytes over the entire time interval assayed, except for the extract from oocytes lacking a meiotic spot after a 10 h incubation (10 h, GVM -). The low H1 histone kinase activity in this sample was presumably due to the failure of some of the pooled oocytes to undergo meiotic maturation. In summary, these experiments showed that GVM and GVBD in the presence of v-ErbA were not caused by the activation of the cAMP-regulated CDC2/MPF pathway.

(2) *v-ErbA initiated changes in the nuclear membrane characteristic of early and intermediate meiotic events*

Many studies have relied solely on inspection of whole oocytes and dissection of nuclei to assay for GVM and GVBD respectively. However, this approach does not

allow the distinction of complete maturation from arrested meiosis which is also accompanied by nuclear destabilization (Baltus *et al.*, 1973; Steinert *et al.*, 1974). To enable me to discriminate between these two outcomes in v-*erbA*-injected oocytes, I performed a detailed analysis by semi-thin sectioning and interference contrast microscopy. Oocytes injected with v-*erbA* expression template showed pronounced morphological changes in the vegetal half of the nuclear membrane which were progressive over time (Fig. 18). It should be noted here that v-*erbA*-injected oocytes appeared fully viable 24 to 36 h after microinjection, as determined by the presence of dark, unmottled pigmentation of the animal hemisphere surrounding the white spot (Fig. 17A), and by comparison with degenerative changes in oocytes of low viability under interference contrast (Fig. 19). Degeneration in these oocytes was marked by the appearance of large vacuoles in the nucleus and cortex. As shown in Fig. 20A, extensive invaginations of the basal nuclear membrane and lamina were visible in v-*erbA*-expressing oocytes 16 h after microinjection. At 36 h, the invaginations appeared as sealed off lacunae of cytoplasm separated by stretches of nuclear membrane, and a yolk-free area was visible in the cytoplasm below (Fig. 20B). At this stage, the projections of nuclear membrane into the nucleoplasm were not restricted to the vegetal half of the nucleus, but could be seen along its circumference. In progesterone-induced maturation, the rupture of the nucleus always occurs in the vegetal half (Baltus *et al.*, 1973; Bement and Capco, 1990; Hausen and Riebesell, 1991). In contrast, in v-*erbA*-injected oocytes, breakdown of the vegetal half of the nuclear membrane was not observed over a 42 h period. Instead, the dynamic rearrangements of the nuclear membrane eventually resulted in membrane rupture at the apical pole after 42 h (Fig. 20C), and

Figure 18. Semi-thin section of a stage VI (Dumont, 1972) *Xenopus* oocyte 36 h after microinjection of 5 ng of v-*erbA* expression construct, viewed under differential interference contrast. N, nucleus; CYT, cytoplasm. Bar: 100 μm.

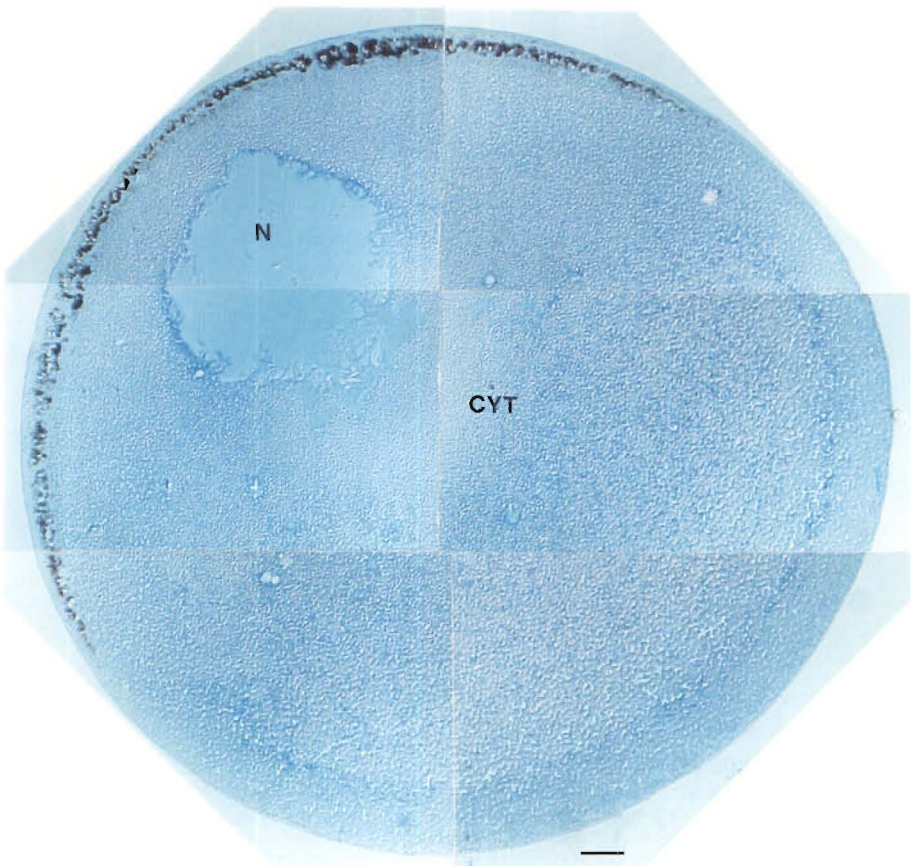
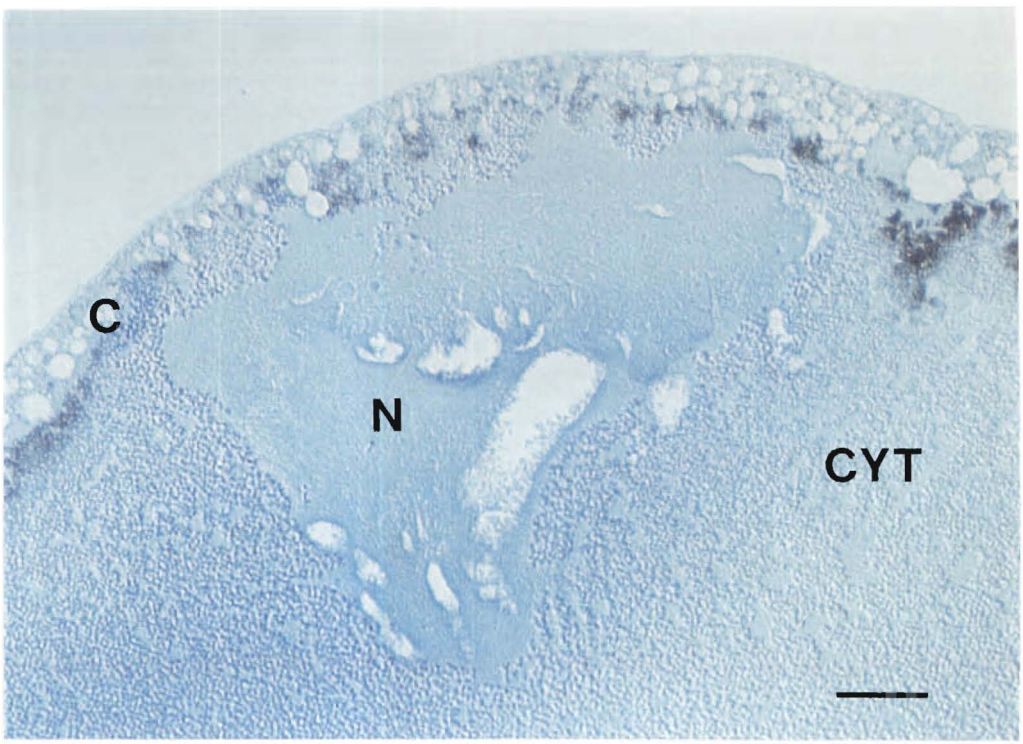

N
CYT

Figure 19. Degenerative changes in a stage V (Dumont, 1972) *Xenopus* oocyte of low viability incubated in O-R2 for 20 h. N, nucleus; CYT, cytoplasm; C, cortex. Bar: 100 μm.


C
N
CYT

subsequent cytolysis at approximately 48 h (data not shown). Apical membrane rupture was not observed in *lacZ*-injected controls, demonstrating that rupture in v-*erbA*-injected oocytes was not due to damage during the process of microinjection (Fig. 20E). Nuclear membrane rupture at the apex, associated with arrested meiotic maturation, has previously been shown to be independent of the site of oocyte microinjection (Baltus *et al.*, 1973; Steinert *et al.*, 1974).

For comparison, progesterone-treated oocytes exhibited large invaginations of the basal nuclear membrane during intermediate meiotic maturation (16 h incubation), and nucleoplasm leaked into the cytoplasmic space, thereby creating a large yolk-free zone (Fig. 20D). After 26 h, meiotic spindle formation had occurred (Fig. 21). These observations showed that the structural alterations in the nuclear membrane of v-*erbA*-expressing oocytes, present 36 h after microinjection, were comparable to the nuclear membrane changes taking place during the intermediate stage of meiosis (Fig. 22). However, the transformations of nuclear membrane architecture occurred more slowly in v-*erbA*-expressing than in progesterone-induced oocytes; and the reorganization of the nuclear envelope was arrested at this intermediate stage in v-erbA-expressing oocytes.

(3) *v-ErbA initiated ultrastructural transformations in the nucleus and the cortical region*

The marked changes in cell ultrastructure that occur during oocyte maturation have been well characterized (Bement and Capco, 1990). Thus, to further investigate the alterations of the nuclear membrane in v-*erbA*-expressing oocytes, I carried out ultrastructural analysis by EM. First, to ensure that any ultrastructural changes

Figure 20. The structural changes in semi-thin sections of nuclei of v-*erbA*-expressing oocytes are indicative of meiotic induction. (*A*) Invaginations of the basal nuclear membrane and lamina in v-*erbA*-expressing oocytes 16 h after microinjection. (*B*) Progressive v-ErbA-mediated structural changes along the circumference of the nuclear membrane, and yolk-free zone below the basal nuclear membrane, 36 h after microinjection. (*C*) Rupture of the apical nuclear membrane (NM) in v-*erbA*-expressing oocytes 42 h after microinjection. (*D*) Invaginations of the basal nuclear membrane and lamina, and yolk-free space, in progesterone-induced oocytes after 16 h. (*E*) Absence of nuclear membrane invaginations in *lacZ*-injected control oocytes 36 h after microinjection. Bars: 100 μm.

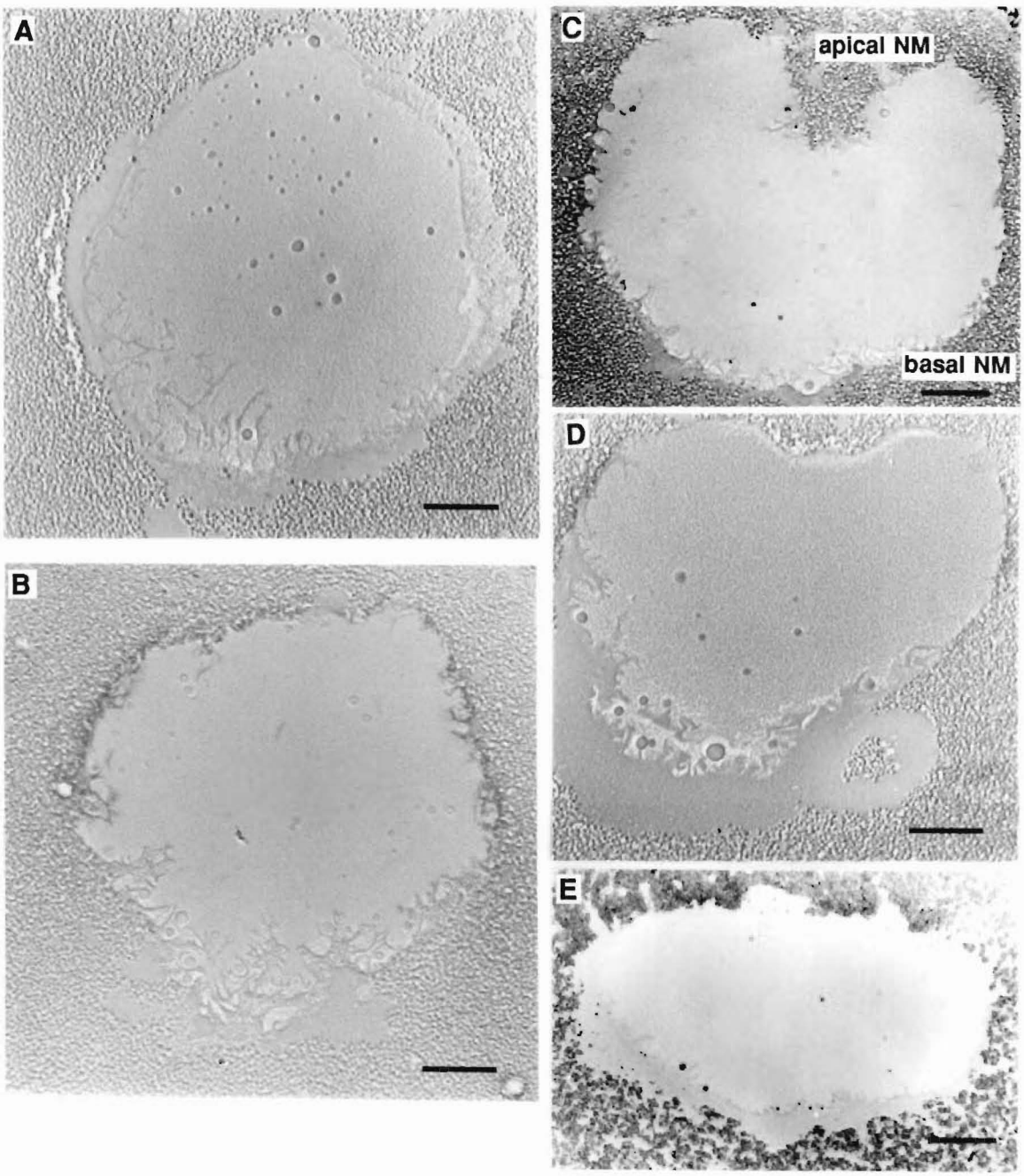

A
B
C
apical NM
basal NM
D
E

Figure 21. Meiotic spindle in a stage VI (Dumont, 1972) *Xenopus* oocyte incubated in progesterone for 26 h. MS, meiotic spindle. Bar: 20 μm.

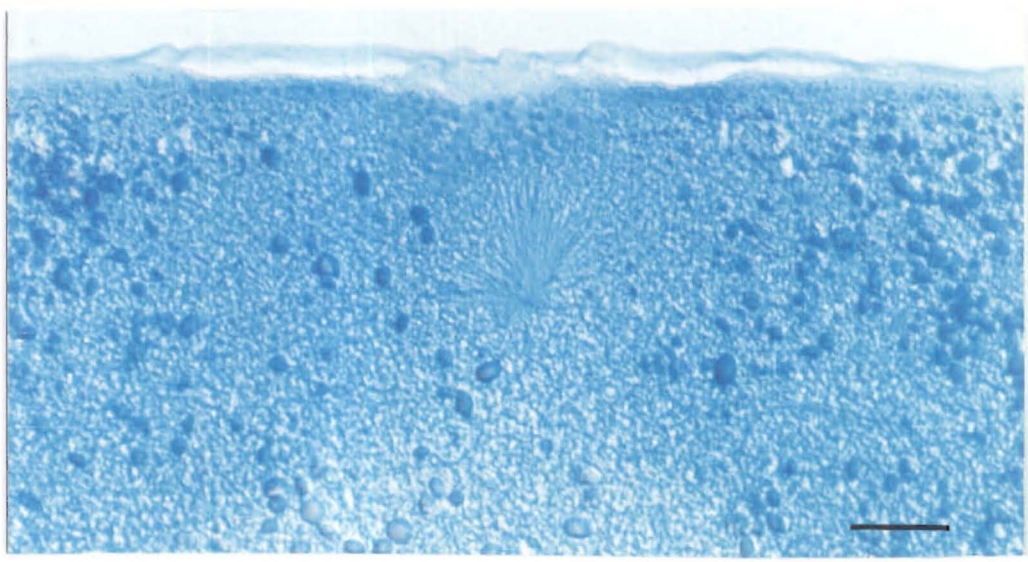
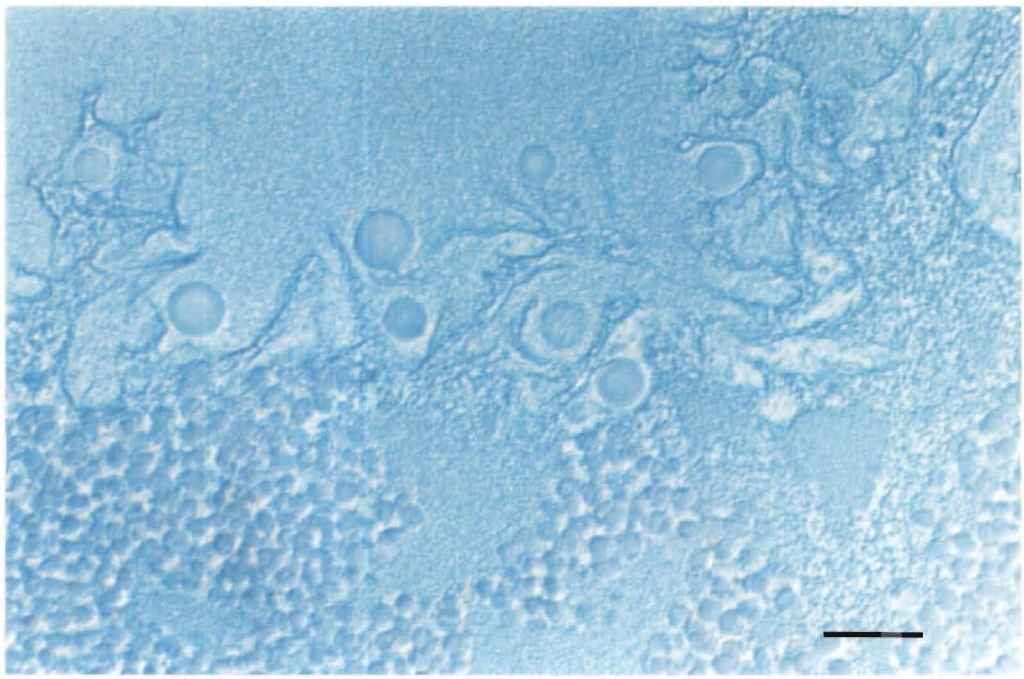
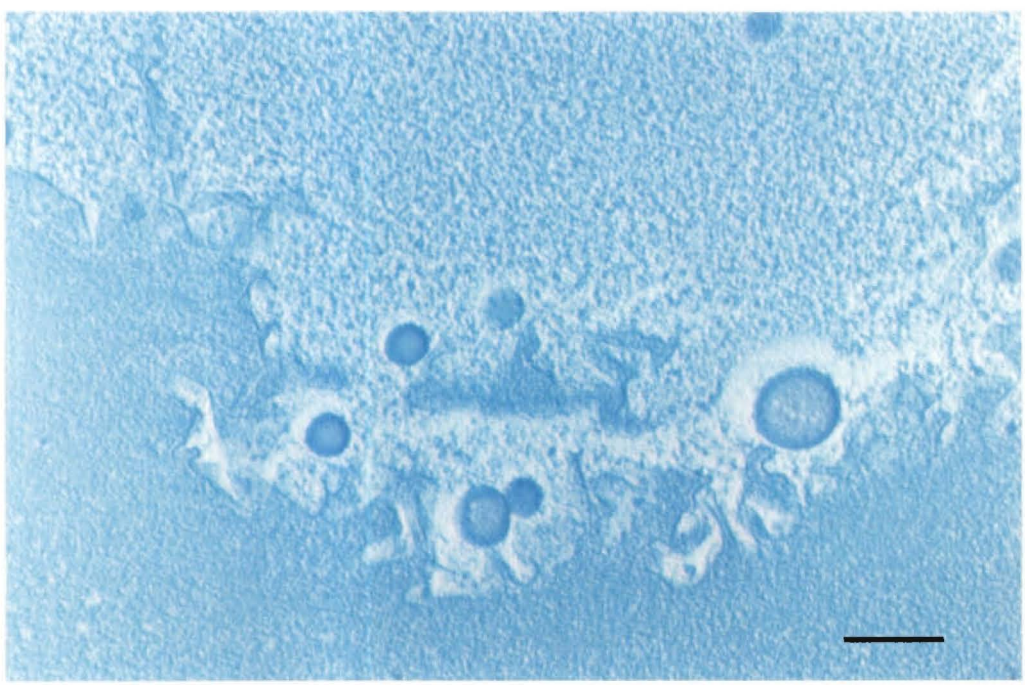


Figure 22. Semi-thin sections of nuclear membranes of stage VI (Dumont, 1972) v-*erbA*-expressing and progesterone-induced oocytes, viewed under high power. Basal nuclear membranes of (*A*) a v-*erbA*-expressing oocyte 36 h after microinjection, and (*B*) an oocyte after a 16 h incubation in progesterone (10 μg/ml). Bars: 20 μm.

A



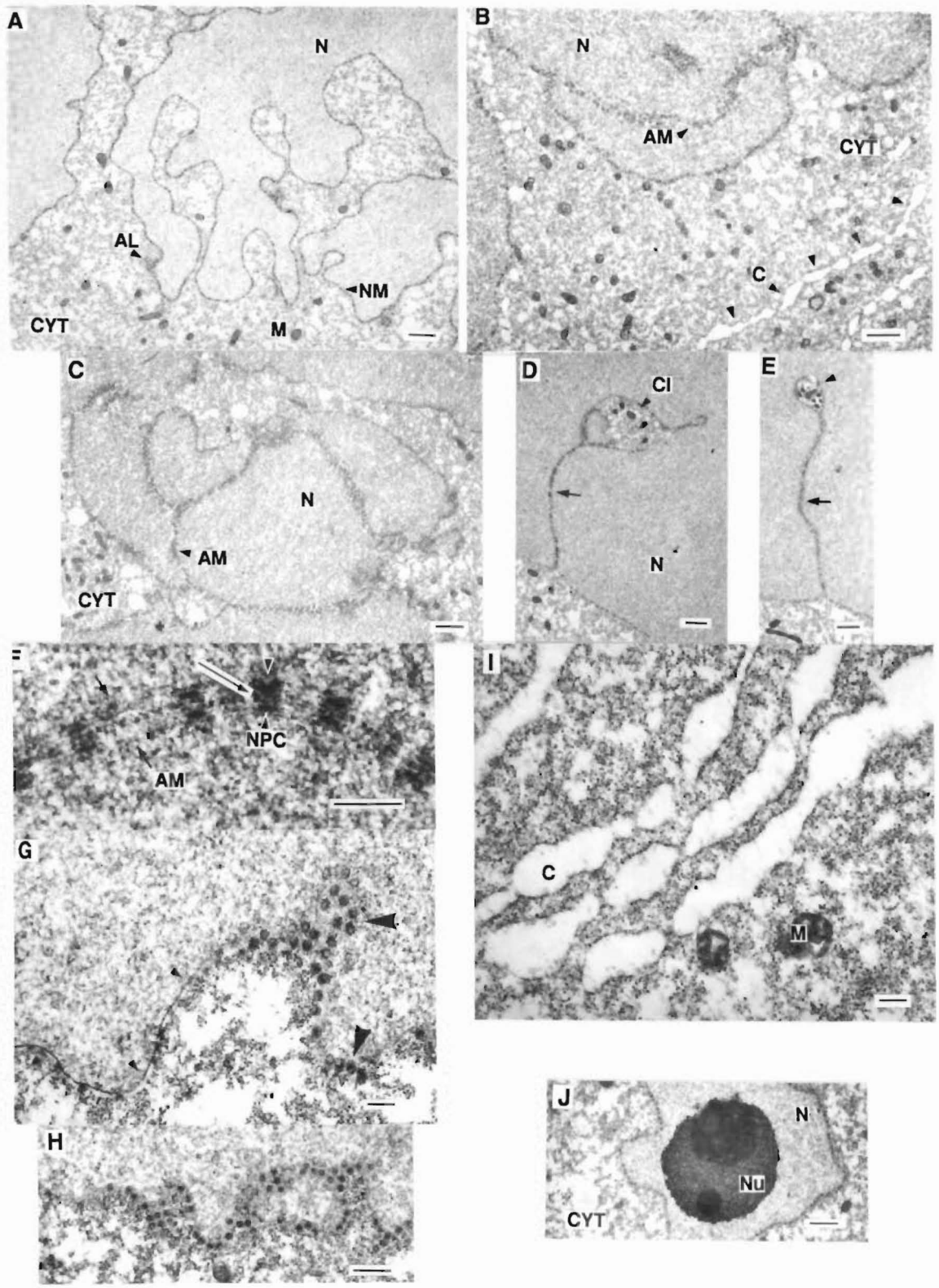
B



observed were not the result of the process of microinjection, I compared *lacZ* template-injected oocytes with v-*erbA*-injected oocytes. *LacZ*-injected oocytes were identical in appearance to uninjected oocytes. The vegetal half of the nucleus was extensively folded, forming narrow cytoplasmic channels and large lobes extending into the nucleoplasm (Fig. 23A). Annulate lamellae (AL) were often seen in the cytoplasm, in close proximity to the nuclear membrane (Fig. 23A).

In contrast, in v-*erbA*-expressing oocytes, the nuclear membrane was drastically altered, while having maintained complete continuity, in the basal region. v-ErbA induced the formation of long stretches of adjoined double membranes and the transformation of AL into cisternae (Fig. 23B-E). Seen in cross-section, the two double membranes appeared tightly adjoined to each other, except for small islands of enclosed cytoplasmic material (Fig. 23D and E). A striking feature of this membrane rearrangement was the alignment of NPCs across the joined membranes (Fig. 23F). High magnification revealed that the cytoplasmic annuli of the NPCs were joined to their counterparts across membranes by electron-dense material. The joined nuclear membranes, when manually isolated from oocyte nuclei, were firmly attached to each other and resistant to separation during tissue processing (data not shown). Furthermore, clustering of NPCs, with adjacent portions of nuclear membrane depleted of NPCs, was seen in the basal regions of the nuclear envelope (Fig. 23G and H). In regions of NPC clustering, projections of nuclear membrane extended into the nucleoplasm. Concomitant with the altered nuclear membrane architecture, multiple layers of cisternae devoid of NPCs developed (Fig. 23I), which typically contain fibrillar material attached to the inner side of the membrane (Kessel and Subtelny, 1981). In addition, the nucleoli showed signs of meiotic

Figure 23. Ultrastructural meiotic changes in thin sections of nuclei of v-*erbA*-expressing oocytes. (*A*) The basal nuclear membrane (NM) of control oocytes injected with 5 ng *lacZ* template was highly folded and formed narrow channels of cytoplasm (CYT) and lobes extending into the nucleus (N). Annulate lamellae (AL) were visible close to the nuclear membrane. M, mitochondrion. (*B* to *J*) Thin sections of v-*erbA*-expressing oocytes. (*B*) and (*C*) Extensive regions of nuclear membrane were tightly adjoined to each other 36 h after microinjection (adjoined membranes, AM). AL had become transformed into cisternae (C). (*D*) and (*E*) Long stretches of adjoined membranes (short arrows) with aligned NPCs (arrowheads) separated off islands of cytoplasm (CI) containing mitochondria and other cytoplasmic material. (*F*) NPCs (arrowheads) were aligned across the adjoined membranes (short arrows) by electron-dense material (long arrow). (*G*) and (*H*) NPCs in the basal nuclear membrane were clustered (large arrowheads), with adjacent regions being depleted of NPCs (small arrowheads), 36 h after microinjection. (*I*) AL were transformed into cisternae (C) devoid of NPCs. (*J*) The nucleoli (Nu) were segregated into distinct fibrillar and granular regions. Bars: (*A*) 1 μm; (*B*) 1 μm; (*C*) 1 μm; (*D*) 1 μm; (*E*) 1 μm; (*F*) 200 nm; (*G*) 200 nm; (*H*) 500 nm; (*I*) 200 nm; (*J*) 1 μm.


A
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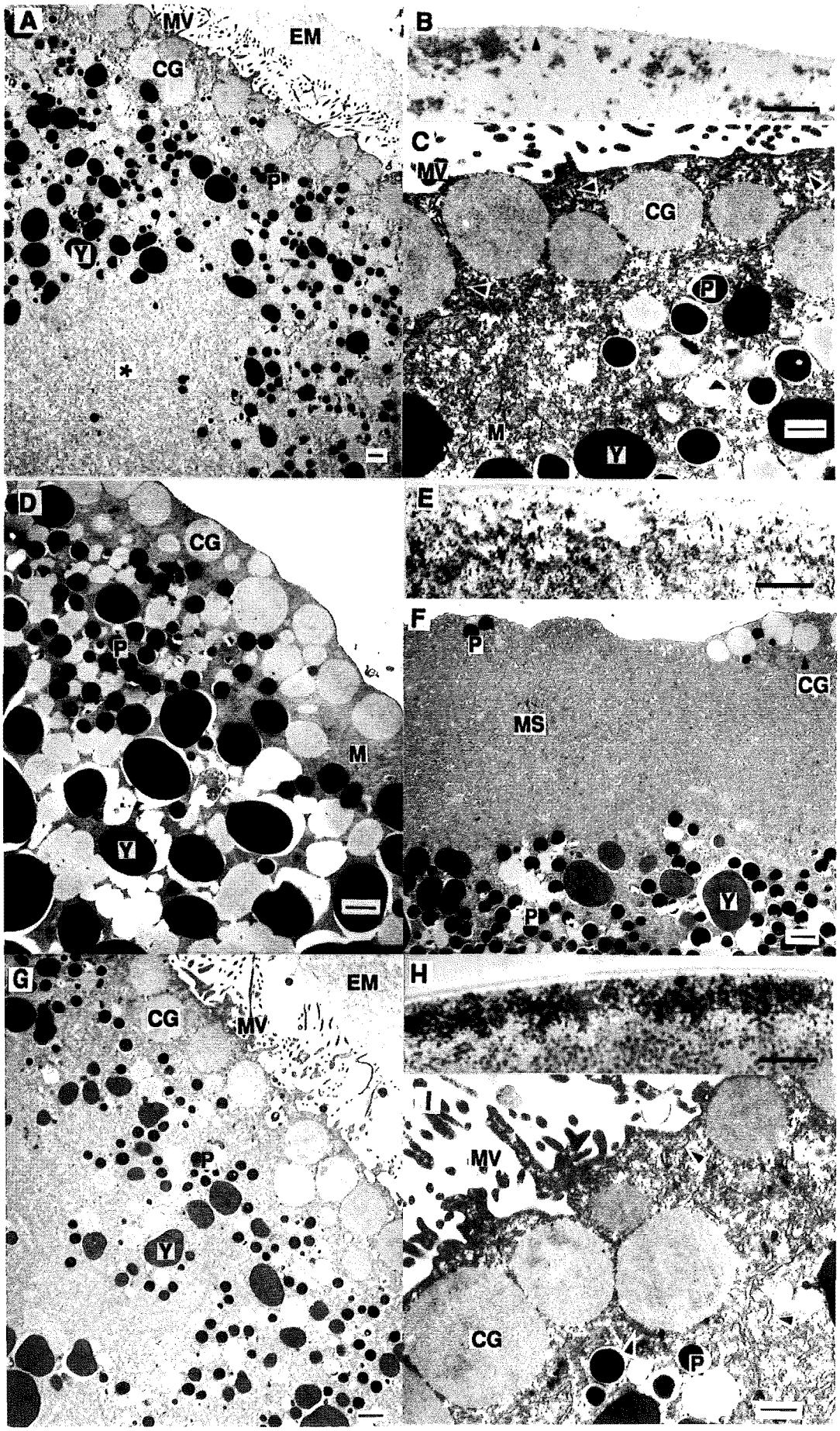
transformation (Fig. 23J), i. e., the granular and fibrillar parts had become clearly segregated (Steinert *et al.*, 1974).

The cortex of v-*erbA*-expressing oocytes exhibited a subset of meiotic changes, but did not develop the characteristics belonging to the late stage of maturation (Fig. 24A-C). Microvilli were present 36 h after microinjection of v-*erbA* template (Fig. 24A and C), while they were lacking in oocytes, undergoing hormone-induced maturation, after 16 h (Fig. 24D). Pigment was clustered at a distance from the plasma membrane in both v-erbA-expressing (Fig. 24B) and progesterone-induced oocytes (Fig. 24E), resulting in the formation of a discrete white spot at the animal pole. In contrast, the cortical granules of v-*erbA*-expressing oocytes remained predominantly localized adjacent to the plasma membrane in the area of the white spot (Fig. 24B), but were retracted into the oocyte interior in progesterone-treated oocytes (Fig. 24E and F). This finding is consistent with the observation that the rapid decrease in cAMP level triggered by progesterone correlates with the movement of cortical granules away from the plasma membrane, suggesting that cortical granule position is controlled by the level of intracellular cAMP (Bement and Capco, 1990). Since v-ErbA-induced changes occur without a decrease in cAMP, the cortical granules would be expected to remain at the plasma membrane. Although the movement of both pigment and cortical granules during maturation is thought to be mediated by an actin network in the oocyte cortex, cortical granules apparently do not become associated with the actin network until after GVBD is complete (reviewed in Bement and Capco, 1990). Our observations provide further evidence for differential control of localization and movement of the two types of granules. In oocytes microinjected with the *lacZ* plasmid, microvilli were more numerous and

Figure 24. The cortex of v-*erbA*-expressing oocytes showed a subset of meiotic transformations in thin and semi-thin sections. (*A*) In the cortex of v-*erbA*-expressing oocytes microvilli (MV) and yolk-free corridors (asterisk) were visible 36 h after microinjection. Cortical granules (CG) were localized immediately below the plasma membrane. EM, extracellular matrix; P, pigment granules; Y, yolk platelet. (*B*) Pigment granules were retracted and clustered in the cytoplasm, while cortical granules (arrowheads) were present adjacent to the apical plasma membrane, in the area of the white spot 36 h after microinjection of v-*erbA* (semi-thin section). (*C*) The microvilli of v-*erbA*-expressing oocytes were partially retracted and of low density. The cortical endoplasmic reticulum (CER) (arrowheads) had not formed an extensive network and did not encircle the cortical granules. (*D*) Progesterone-induced maturation resulted in the complete retraction of microvilli after 16 h; yolk-free corridors were disrupted, and mitochondria (M) were randomly dispersed throughout the cortex. (*E*) Progesterone induction caused the retraction of cortical granules and the clustering of pigment granules in the cytoplasm (semi-thin section). (*F*) Retraction of almost all cortical granules, pigment granules, and other vesicles, resulted in an area of clear cytoplasm in the meiotic white spot (MS) of progesterone-induced oocytes after 16 h. (*G*) Cortex of a *lacZ*-injected control oocyte 36 h after microinjection. (*H*) Continuous pigment layer adjacent to plasma membrane in *lacZ*-injected oocyte 36 h after microinjection (semi-thin section). (*I*) Numerous extended microvilli (MV) in *lacZ*-injected oocyte 36 h after microinjection. The CER (arrowheads) was comparable to that of v-*erbA*-injected oocytes. Bars: (*A*) 1 μm; (*B*) 100 μm; (*C*) 500 nm; (*D*) 1 μm; (*E*) 100 μm; (*F*) 1 μm; (*G*) 1 μm; (*H*) 100 μm; (*I*) 500 nm.


A
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*
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CG
P

further extended (Fig. 24G and I), than in v-*erbA*-injected oocytes (Fig. 24A and C). The layer of pigment granules was continuous in *lacZ*-injected oocytes and located directly adjacent to the plasma membrane (Fig. 24H), differing markedly from the clustered pigment arrangement in v-*erbA*-injected oocytes (Fig. 24B). Finally, two characteristic features of late maturation, disruption of the yolk-free corridors and development of an extensive cortical endoplasmic reticulum, were not seen in v-*erbA*-expressing oocytes (Fig. 24A and C). The structure of the cortical endoplasmic reticulum in *lacZ*-injected controls (Fig. 24I) was comparable to that of the cortical endoplasmic reticulum in v-*erbA*-expressing oocytes (Fig. 24C). In conclusion, structural analysis provides convincing evidence that v-ErbA induces early to intermediate meiotic events in *Xenopus* oocytes. A summary of the comparison of ultrastructural changes induced by progesterone and v-ErbA is presented in Table II.

Table II. Induction of early and intermediate meiotic events by v-ErbA in stage VI (Dumont, 1972) *Xenopus* oocytes.

	Inducer	
	progesterone[1]	v-ErbA[2]
Timing of meiotic events[3]		
early	AL transformation	+
	basal membrane changes	+
	cortical granule retraction	-
intermediate	pigment rearrangement	+
	NPC repositioning	initiated
	nuclear migration	+
	nucleolar dissolution	initiated
	microvilli retraction	initiated
	GVBD	apical breakdown
	chromosome condensation	-
late	mitochondrial corridor disruption	-
	cortical ER development	-
	microvilli retraction completed	-
	meiotic spindle	-

[1] Maturation events were identified according to Bement and Capco (1990); Brachet *et al.* (1970); Kessel and Subtelny (1981); Kessel (1992); and Steinert *et al.* (1974).

[2] The presence (+) or absence (-) of events, indicative of different stages of progesterone-induced maturation, in v-*erbA*-expressing oocytes is shown (see Figs. 23 and 24).

[3] Meiotic events were timed as described in Bement and Capco (1990).

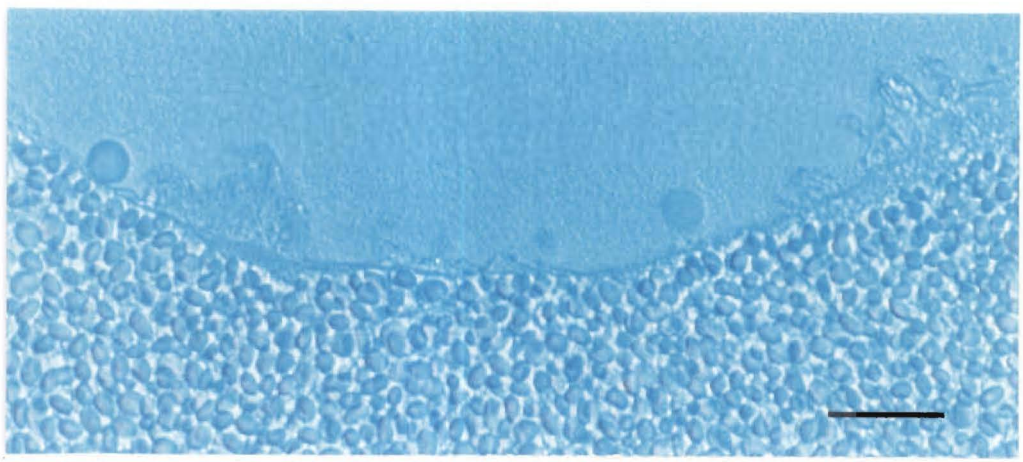
(4) *v-ErbA acted at the level of gene activation*

Some of the growth-promoting properties of v-ErbA are mediated by dominant repression of TR and RAR (Desbois *et al.*, 1991a, 1991b; Zhang *et al.*, 1991; Gandrillon *et al.*, 1994; Sharif and Privalsky, 1991). Stage VI *Xenopus* oocytes contain low levels of TRα and TRß protein (Eliceiri and Brown, 1994), but lack thyroid hormone (T_3) (Chapter II; Nagl *et al.*, 1995). Conceivably, unliganded TR might contribute to the G_2 arrest by repressing certain M-phase-inducing genes. Therefore, I wished to assess whether dominant negative inactivation of unliganded TR by v-ErbA was responsible for the induction of early meiotic events. I have shown in Chapter II that the action of endogenous oocyte TR can be blocked by a dominant negative *in vitro*-generated mutant of human TRß (TR C122>A) (Nagl *et al.*, 1995). This DNA-binding deficient mutant is thought to block TR-mediated gene regulation by forming inactive dimers with TR (Nelson *et al.*, 1993). When v-ErbA was replaced by the mutant TR C122>A, the morphology of the basal nuclear membrane in TR C122>A-injected oocytes was identical to that of untreated G_2-arrested oocytes (Fig. 25A). This finding suggested that v-ErbA did not initiate maturation events by acting as a dominant antagonist of endogenous TR and pointed to a direct role in gene activation for v-ErbA.

Thus, to test whether release of the G_2 arrest by v-ErbA required *de novo* gene expression, the frequency of GVBD was scored in oocytes that had been injected with *in vitro*-synthesized v-*erbA* protein and incubated in actinomycin D, an inhibitor of transcription, or cycloheximide, an inhibitor of protein synthesis. This experiment was carried out in collaboration with Caroline F. Bunn. Since v-*ErbA* was not purified from the translation reaction mixture, it was possible that the rabbit

Figure 25. v-ErbA acts at the level of gene activation, but not as an antagonist of TR. (*A*) A dominant negative mutant of TR has no effect on nuclear structure. The basal nuclear membrane of a TR C122>A-injected oocyte 36 h after microinjection. Bar: 20 μm. (*B*) GVBD initiation by v-ErbA required *de novo* gene expression. The frequency of GVBD was scored by dissection of nuclei from uninjected controls, or from oocytes injected with *in vitro*-synthesized v-ErbA (in rabbit reticulocyte lysate), after a 24 h incubation in O-R2, 30 μg/ml actinomycin D (Act. D), or 200 μg/ml cycloheximide (CXD). As an additional control, oocytes were injected with the rabbit reticulocyte lysate transcription/translation mixture (TNT) and scored for GVBD. The error bars indicate the SEMs of 3 independent experiments (30-40 oocytes/treatment/experiment).

A



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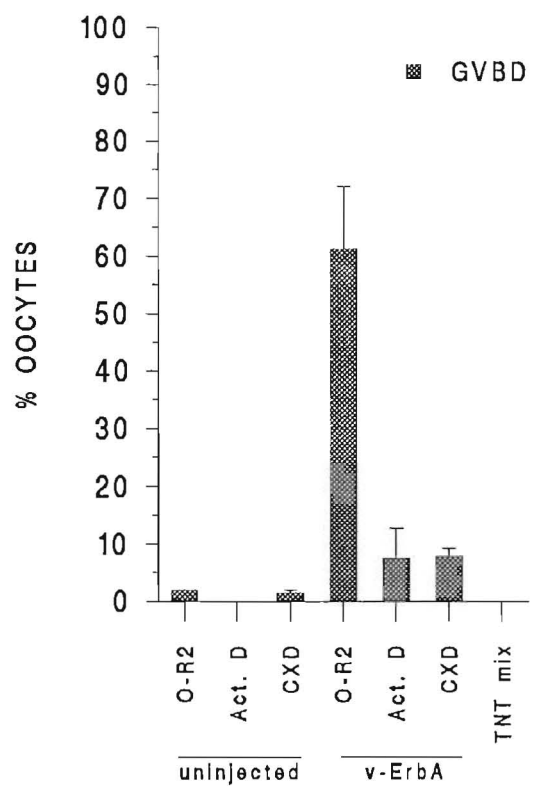
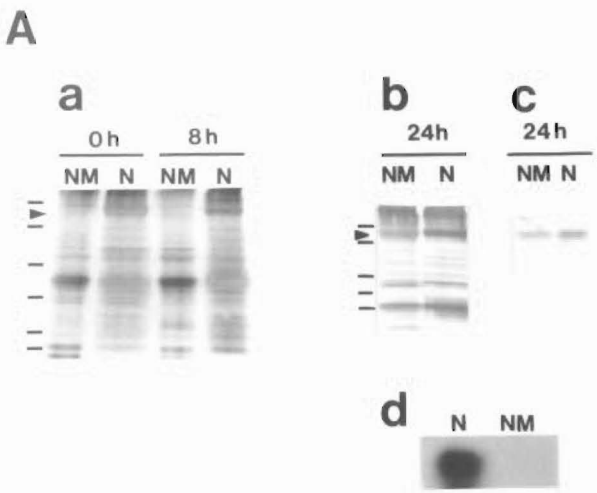

100
90
80
70
60
50
40
30
20
10
0
% OOCYTES
GVBD
O-R2
Act. D
CXD
O-R2
Act. D
CXD
TNT mix
uninjected
v-ErbA

reticulocyte lysate could supply factors that would effect GVBD. Thus, oocytes were injected with lysate mixture as a control. As shown in Fig. 25B, the lysate alone did not induce GVBD. Twenty-four hours after microinjection, the mean frequency of GVBD in oocytes injected with v-ErbA, and incubated in the absence of inhibitors, was 61% (Fig. 25B). Incubation of v-ErbA-injected oocytes in either actinomycin D or cycloheximide significantly reduced the frequency of GVBD to 7.5% (Fig. 25B), showing that meiotic induction by v-ErbA is dependent on mRNA synthesis and translation. Taken together, these results suggest that v-ErbA initiates meiotic events by activating a key regulator gene(s) in oocytes.

(5) A portion of v-ErbA was present at nuclear pore complexes

Srp1, a *Saccharomyces cerevisiae* homolog of importin, the vertebrate nuclear localization sequence (NLS) receptor, is essential for nuclear protein import, and is required for mitosis (Loeb *et al.*, 1995). Conditional *srp1* mutants arrest in the cell cycle during the G_2/M phase, suggesting that import of cell cycle regulators into the nucleus is critical for cell cycle progression. Thus, it was of interest to determine whether v-ErbA associated with NPCs, thereby potentially modulating nucleocytoplasmic transport of cell cycle regulators. In immunoprecipitation assays, the majority of v-*erbA* protein, expressed from the RS-v-erbA construct, is recovered from manually isolated oocyte nuclei, while approximately 10% of v-ErbA partitions to the cytoplasmic fraction (Chapter II). As shown in Fig. 26A, a protein with a molecular weight of approximately 75 kD, corresponding in size to the full-length *gag*-v-*erbA* fusion protein encoded by the RS-v-erbA expression template (Privalsky, 1992), was predominantly detected in total soluble protein fractions from nuclei 8 h

Figure 26. A portion of v-ErbA was present at NPCs. (*A*) Low amounts of v-ErbA were associated with the nuclear membrane. *Panel a*: Total soluble protein fractions were prepared from 10 manually isolated nuclei (N) or nuclear membranes (NM), 0 or 8 h after microinjection of 5 ng v-*erbA* expression template, and resolved by 12% SDS-PAGE and silver staining. The arrowhead indicates a protein of approximately 75 kD, corresponding in size to the full-length *gag*-v-*erbA* fusion protein. Molecular weights (D) are indicated by dashes: 85,200; 55,600; 39,200; 26,600; 20,100; 14,300. *Panel b*: Total soluble protein fractions from 10 nuclei or 10 nuclear membranes 24 h after microinjection. Symbols are as described in *Panel a*. *Panel c*: The 75 kD *gag*-v-*erbA* protein was immunoprecipitated from the nuclear membrane and nuclear fractions using anti-v/c-*erbA* antibodies. Oocytes microinjected with 5 ng *v-erbA* expression vector were cultured in O-R2 medium with 1 mCi/ml L-[^{35}S] methionine for 24 h, and nuclear membranes and nuclei were manually isolated. Nuclear membrane (NM) and nuclear (N) fractions of 10 *v-erbA*-injected oocytes were incubated with protein G-Sepharose-antibody complexes in an immunoprecipitation assay. Labelled polypeptides were recovered and separated by 12% SDS-PAGE followed by fluorography. *Panel d*: c-ErbA was exclusively immunoprecipitated from the nuclear fraction. Immunoprecipitation of c-*erbA* protein from the nuclear and nuclear membrane fractions of 20 oocytes injected with 5 ng c-*erbA* expression vector was carried out as described in *Panel c*. (*B*) Immunogold EM detection of v-ErbA at NPCs. Manually isolated nuclear membranes from v-*erbA*-injected oocytes were immunolabelled with 10-nm colloidal gold, using a v-*erbA*-specific monoclonal antibody in a biotin-streptavidin bridging technique, prior to embedding and thin sectioning. *Panel a*: An en face view of the cytoplamic side of the nuclear envelope. Arrowheads indicate examples of colloidal gold-labelled NPCs clearly revealed by this plane of sectioning. *Panels b* and *c*: Selected examples of transversely sectioned NPCs labelled by the anti-v-*erbA* antibody. *Panels d* and *e*: Representative examples of tangentially sectioned nuclear envelopes. The v-*erbA*-specific antibody exclusively labelled the cytoplasmic fibrils of NPCs. The cytoplasmic side of all cross-sectioned nuclear envelopes faces the top of the figure. The cytoplasmic side of the nuclear envelope often forms blebs (*Panel e*). Bars: *Panel a*, 200 nm; *Panel b*, 100 nm; *Panels c-e*, 50 nm.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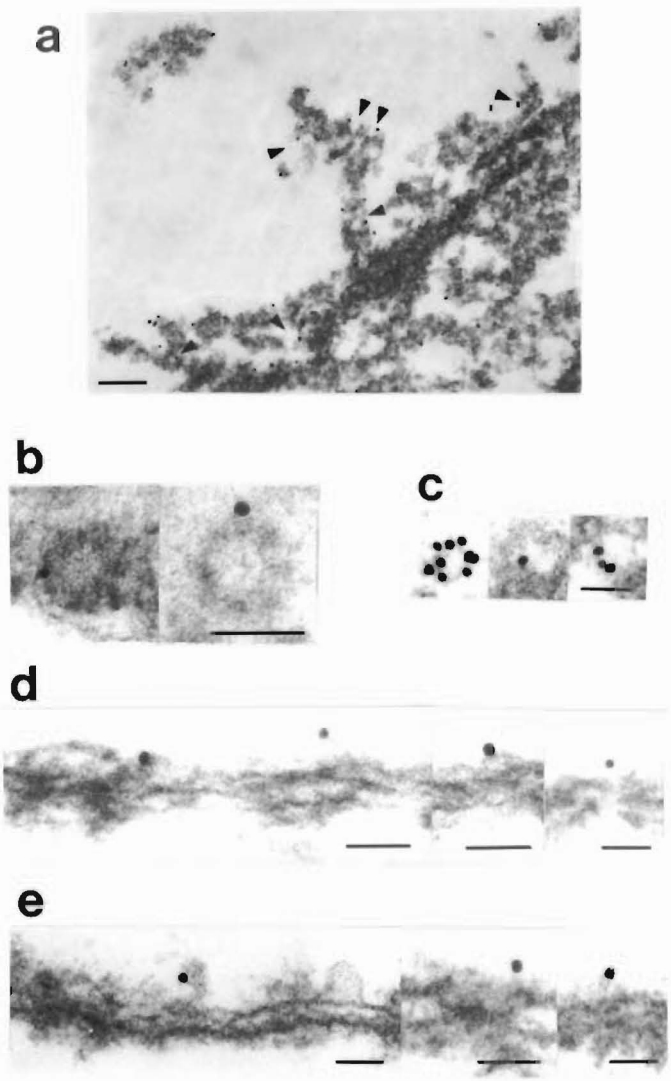
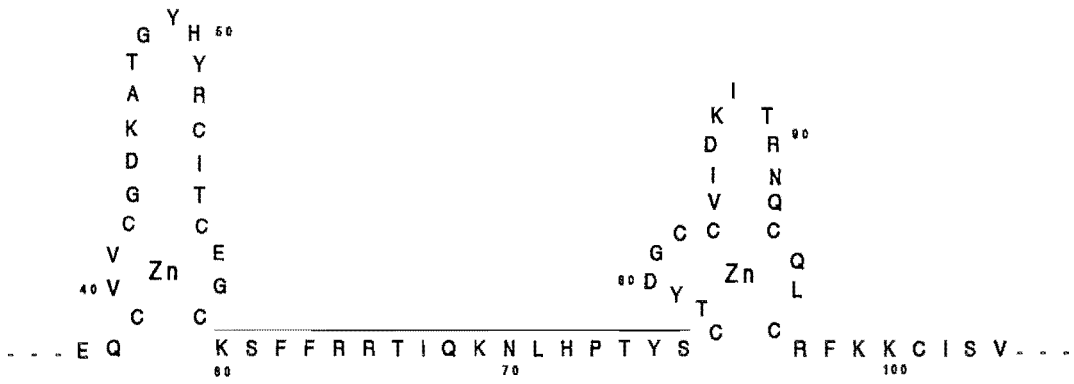


after microinjection of the gene template (*Panel a*). Interestingly, low levels of a protein of comparable size were also detectable in total soluble protein fractions from nuclear membranes after 8 h (*Panel a*). After 24 h, v-ErbA accumulation in both the nuclear and nuclear membrane fraction was further increased (*Panel b*). In addition, v-ErbA was immunoprecipitated from both fractions by a monoclonal antibody with dual specificity for both v-*erbA* and chicken c-*erbA* (Freake *et al.*, 1988) (*Panel c*). In contrast, deliberate overexposure of the fluorograph showed that wild-type chicken c-ErbA, expressed from injected c-*erbA* templates, was exclusively immunoprecipitated from the nuclear fraction (Panel *d*).

To identify the precise location of the v-*erbA* product, I performed immunogold labelling of manually isolated nuclear membranes (Fig. 26B). Nuclear membranes, isolated from v-*erbA*-expressing oocytes, were exclusively decorated with gold particles at the cytoplasmic face of NPCs (*Panels a* to *c*) and, more specifically, the v-*erbA* product was localized to the fibrils emanating from the cytoplasmic annuli (*Panels d* and *e*). A possible reason for the relatively low labelling density, which contrasted with the strong signal obtained by immunoprecipitation, is that the primary antibody was raised against a synthetic peptide of v-*erbA* (residues 58-75) within the DNA binding domain. Since the v/c-*erbA* NLS is separated from the DNA binding domain by only 12 amino acid residues (Fig. 27) (Dingwall and Laskey, 1991; Lacasse and Lefebvre, 1995), antibody accessibility might have been reduced due to steric hindrance between the DNA binding domain and a NPC component(s) interacting with the v-ErbA NLS. In summary, immunogold localization of v-ErbA to the cytoplasmic fibrils of NPCs points to the possibility that this oncogenic protein might mediate some of its effects by interfering with NPC function.

Figure 27. Amino acid sequence of the epitope within the DNA binding domain (residues 58-75) and the nuclear localization signal of v-ErbA. v-ErbA residues 58-75 (indicated by a line), against which the monoclonal antibody LA038 was raised, are part of the DNA binding domain. The nuclear localization signal (NLS) (boxed) is separated from the DNA binding domain by only 12 amino acid residues.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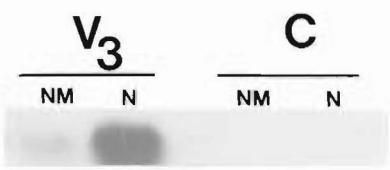
- - - E Q
K S F F R R T I Q K N L H P T Y S
R F K K C I S V - - -
60
70
100


- - - K R V A K R R K L I E E N R E R R R - - -
120
130

(6) ***Preliminary results suggest that the association with NPCs might be mediated by the gag protein***

The v-*erbA* oncogene is expressed as a fusion product with the viral *gag* sequence (Privalsky, 1992). The results described in the previous section pointed to a possible involvement of *gag* in mediating the association of v-ErbA with NPCs, since it was shown that c-ErbA alone was not localized to the nuclear membrane. Interestingly, in preliminary experiments, a chimeric *gag*-c-*erbA* product (V_3), was also immunoprecipitated from isolated nuclear membranes (Fig. 28). This finding supports the notion that the *gag* portion of the *gag*-v-*erbA* product might be mediating association with NPCs. Since NLSs have been identified in the *gag* products of some retroviruses, such as human immunodeficiency virus 1 (HIV-1), and foamy viruses (Bukrinsky *et al.*, 1993; Schliephake and Rethwilm, 1994), I analyzed the *gag* sequence of the *gag*-v-*erbA* fusion product for sequence homologies with known NLS motifs (LaCasse and Lefebvre, 1995; Siomi and Dreyfuss, 1995). However, as shown in Fig. 29, the AEV *gag* sequence contains only dispersed hydrophobic residues, and does not exhibit any previously identified NLS motif. Therefore, it seems unlikely that the observed *gag*-mediated association of the *gag*-v-*erbA* protein with NPCs was caused by Gag interactions with a NLS receptor. On the other hand, the retroviral Gag proteins have been termed "particle-making machines", since they direct the formation of virion-like particles through Gag-Gag interactions, even when expressed in the absence of all other virus-encoded components (reviewed in Wills and Craven, 1991). It is therefore possible that Gag-Gag interactions between several *gag*-v-*erbA* proteins, simultaneously docked at the same NPC, caused the aggregation of these proteins and thereby blocked their

Figure 28. Immunoprecipitation of the *gag*-c-*erbA* product (V_3) from nuclear and nuclear membrane fractions. Oocytes injected with 5 ng V_3 mRNA into the cytoplasm (V_3), or uninjected control oocytes (C), were incubated in O-R2 medium with 1 mCi/ml L-[^{35}S] methionine for 24 h, and nuclear membranes and nuclei were manually isolated. Nuclear membrane (NM) and nuclear (N) fractions of 20 oocytes per sample were prepared and processed as described in Fig. 26.


V3
C
NM
N
NM
N

5'-REEQVTSEQAKFWLGLGGGRVSPPGPECIEKPATERRIDKGEMGETTVQRDA KMAPEKMATPKTVGTSCYQCGTATGCNCVTASAPPPPYVGSGLTPSLAGAGEQ GQGGDTPRGAEQPRAEPGHAGQAPGPALTDWARIREELASTGPPVVAMPVVIL TEGPAWTPLEP-3'

Figure 29. AEV *gag* amino acid sequence sequence (GeneBank Accession Number M32090 Y00044). Single letter amino acid code is used and hydrophobic amino acids (lysine, K; arginine, R) are underlined. Nucleotide sequence conversion was carried out by DNASIS open reading frame display.

nuclear import. The putative *gag*-dependence of v-ErbA association with NPCs was not investigated further, since it lies outside the scope of this investigation. It does, however, raise the interesting question of whether the Gag portion of the *gag*-v-*erbA* fusion protein, although it is not involved in transcriptional regulation by v-ErbA (Sap *et al.*, 1989), might nevertheless contribute to the function of this oncogenic protein by affecting its subcellular localization.

4. Discussion

A detailed understanding of the role of oncogenes in meiotic induction, and in cell cycle control in general, requires the dissection of the complex processes through which subsets of biochemical signal cascades result in distinct structural reorganizations of the cell. My study identified a new role for the retroviral v-*erbA*

protein in partially lifting the cell cycle arrest at the G_2/M transition in *Xenopus* oocytes, and, moreover, characterized the distinct subset of structural transformations induced by v-ErbA.

(1) *v-ErbA induces a subset of meiotic events in Xenopus oocytes independently of CDC2/MPF kinase*

The progressive changes in nuclear structure during oocyte maturation can serve as markers for temporal subsets of meiotic transformations. In the present study, I showed that v-*erbA*-expressing *Xenopus* oocytes exhibited structural changes associated with a subset of meiotic events occurring prior to chromatin condensation. These changes showed remarkable similarity to progesterone-induced events, despite key differences in the mode of induction. Early and intermediate meiotic events were induced by v-ErbA independently of the cAMP-regulated CDC2/MPF pathway, and required gene transcription. In contrast, the induction of *Xenopus* oocyte maturation by progesterone does not require gene transcription, but is absolutely dependent on polyadenylation and translation of stored mRNA coding for the serine-threonine kinase Mos (Sagata *et al.*, 1988; Yew *et al.*, 1992; Sheets *et al.*, 1995).

The identification of a distinct subset of meiotic characteristics induced by v-ErbA is in agreement with other studies, reviewed in the introduction to this chapter, which have shown that meiotic events can be biochemically separated (Baltus *et al.*, 1973; Drury and Schorderet-Slatkine, 1975; Newport and Spann, 1987; Bement and Capco, 1990; Choi *et al.*, 1991; Gavin *et al.*, 1992, 1994; Muramatsu *et al.*, 1989; Waldmann *et al.*, 1990). In addition, NIMA kinase induces nuclear breakdown, pigment rearrangement and chromatin condensation in *Xenopus* oocytes, but not

spindle formation or nuclear migration, and does not activate CDC2/MPF or MAPK (Lu and Hunter, 1995). Furthermore, the membrane-bound guanine nucleotide-binding protein Ras can induce maturation and activation of MAPK by both Mos-dependent and Mos-independent pathways, and can bypass changes in intracellular cAMP levels (Smith, 1989, and refs. therein; Hattori *et al.*, 1992; Pomerance *et al.*, 1992; Shibuya *et al.*, 1992; Nebreda *et al.*, 1993). Recently, it has been shown that cAMP-dependent protein kinase A inhibition of meiosis acts at more than one site, demonstrating that MAPK and MPF activation are not necessarily interdependent events during maturation (Matten *et al.*, 1994). Thus, the changes associated with M-phase induction can be grouped into partially overlapping sets of events which are regulated by several complementary control pathways.

(2) *v-ErbA releases the G_2 arrest in oocytes, but meiosis is blocked at an intermediate stage*

The rearrangements of the basal nuclear membrane, NPC clustering, AL transformation into cisternae, and segregration of the granular and fibrillar components of nucleoli, which were all observed in v-*erbA*-expressing oocytes, represent the initiation of events leading to the dissassembly of the nuclear membrane and NPCs during meiosis (Bement and Capco, 1990; Kessel and Subtelny, 1981; Brachet *et al.*, 1970; Steinert *et al.*, 1974). However, the reorganization of the nuclear membrane in v-*erbA*-injected oocytes did not proceed past this intermediate stage, chromosomes remained decondensed, and eventually the nuclear membrane ruptured at the apical pole. Thus, although nuclear breakdown occurred, the endpoint of the v-*erbA*-induced pathway was cytolysis, rather than meiotic spindle formation.

CDC2/MPF, either directly or through activation of other protein kinases, induces the disassembly of the nuclear lamina filaments and NPCs, and the vesicularization of the nuclear membrane (reviewed in Bement and Capco, 1990; Smith, 1989); and chromosome condensation and meiotic spindle formation are mediated by the reorganization of microtubules by CDC2/MPF and MAPK (Gotoh *et al.*, 1991; Bement and Capco, 1990). The failure of these progressive events to occur in v-*erbA*-expressing oocytes was correlated with the fact that CDC2/MPF kinase was not activated, and that v-ErbA-mediated nuclear changes were not inhibited by taxol. This suggests that the nuclear changes did not involve rearrangements of the microtubular network and, by implication, occurred independently of a MPF/MAPK requirement. NIMA-related kinases, which are thought to be substrates of CDC2, are also involved in the regulation of chromosome condensation (Osmani *et al.*, 1991a, 1991b; O'Connell *et al.*, 1994; Lu and Hunter, 1995; Ye *et al.*, 1995; reviewed in Fry and Nigg, 1995). Interestingly, lack of *nimA* during mitosis causes the proliferation of endomembranes in and around nuclei and fragmentation of the nuclear membrane in *Aspergillus nidulans* (Osmani *et al.*, 1991a). My findings suggest that release of the G_2 arrest by v-ErbA allows meiotic reinitiation up to a point where signal transmission by activated MPF, MAPK, and possibly NIMA-related protein kinases, becomes essential for M-phase progression.

There was a striking match between the v-*erbA*-induced events and the characteristic features seen in the induction of early maturation by the simultaneous treatment of oocytes with progesterone and cycloheximide, or by injection of extract prepared from progesterone-treated oocytes (Baltus *et al.*, 1973; Steinert *et al.*, 1974; Smith, 1989). This subset of structural changes, termed pseudomaturation, is induced

in the absence of *de novo* synthesis of c-*mos* protein and MPF activation (Drury and Schorderet-Slatkine, 1975; Wasserman and Masui, 1975; Kobayashi *et al.*, 1991), and includes adjoined nuclear membranes, mottling of the pigment layer, and condensation of the fibrillar core of the nucleoli (Baltus *et al.*, 1973; Steinert *et al.*, 1974). Moreover, chromosome condensation is never observed in pseudomaturation, and the eventual apical rupture of the nuclear membrane results in cytolysis (Brachet *et al.*, 1970; Steinert *et al.*, 1974; Drury and Schorderet-Slatkine, 1975). Induction of early maturation by microinjected, *in vitro*-synthesized v-ErbA, which required mRNA synthesis and translation, and induction of pseudomaturation in the absence of protein synthesis, appear to constitute two alternate pathways capable of initiating an overlapping set of events.

In oocytes, M-phase induction by v-ErbA, like pseudomaturation, is ultimately a destructive process. The reason for this most likely lies in the absence of additional signals from activated protein kinases, essential for the completion of maturation. Once the G_2/M restriction point is passed, the maturing oocyte is committed to undergo events leading to GVBD and lacks the ability to reverse the meiotic pathway. Consequently, the initiated structural transformations eventually lead to the disintegration of the oocyte. This situation is wholly different from the context in which v-ErbA functions in somatic cells. Somatic cells constantly receive a wide range of extracellular signals conveyed by growth factors, which activate mitogenic kinases (reviewed in Weinberg, 1989a). Abnormal activation of a gene(s), encoding a cell cycle inducer, by v-ErbA can be expected to act in a complementary manner to these events and enhance certain mitogenic signals. As a result, v-ErbA apparently does not cause nuclear dissolution in somatic cells, but confers a reduced requirement

for growth factors and an enhanced growth potential (Gandrillon *et al.*, 1987). These effects of v-ErbA are further discussed in Chapter IV.

(3) *Growth-promoting properties of v-ErbA*

The question of whether the growth-promoting properties of v-ErbA can be exclusively attributed to dominant repression of TR and RAR, or whether it also requires the activation of specific v-ErbA-responsive genes, remains unanswered to date. My study showed that the release of the cell cycle arrest at the G_2/M transition point by v-ErbA required *de novo* gene expression, and occurred independently of cAMP regulation and CDC2/MPF kinase activation. Furthermore, v-ErbA apparently does not act by dominantly inhibiting endogenous TR, since a dominant negative mutant of TR had no effect on cell ultrastructure. Therefore, the putative target gene(s) is unlikely to be regulated by TR in oocytes.

Importantly, the cellular forms of several viral oncogenes are found as signalling components of the MAPK cascade, which suggests that this pathway mediates somatic cell transformation (reviewed in Davis, 1993; Mordret, 1993; Mansour *et al.*, 1994). Within the nucleus, signal transduction by MAPK activates a range of transcription factors by phosphorylation, including the products of the c-*jun* and c-*fos* proto-oncogenes which form the so-called AP-1 complex (Mordret, 1993; Davis, 1993; Avruch *et al.*, 1994). Conceivably, v-ErbA action might mimic the function of a MAPK-activated transcription factor in oocytes. It is interesting to note in this context that another member of the steroid/thyroid receptor superfamily, the oestrogen receptor, was recently shown to be phosphorylated by MAPK *in vitro* (Kato *et al.*, 1995). Thus, v-ErbA might initiate maturation events by activating a

key gene target(s) not normally expressed in the oocyte, which is capable of triggering the release from G_2 arrest and inducing a specific subset of structural transformations.

The ability of a cell to overcome dependence on growth factors, either partially or completely, and to grow in an autonomous fashion, forms the basis of oncogenic transformation. Previously, v-ErbA was shown to confer partial growth factor autonomy by, firstly, rendering cells unresponsive to a range of anti-mitogenic controls; i. e., v-ErbA abolishes AP-1 repression, induction of apoptosis, and self-renewal inhibition mediated by TR and RAR in erythroid precursors (Desbois *et al.*, 1991a and 1991b; Zhang *et al.*, 1991; Sharif and Privalsky, 1991; Saatcioglu *et al.*, 1993b; Gandrillon *et al.*, 1994). Secondly, v-ErbA can activate an indirect autocrine pathway by inducing the platelet-derived growth factor B/c-*sis* gene in glial cells, again by exerting a regulatory role opposite to that of TR (Iglesias *et al.*, 1995). My study suggests that v-ErbA, acting as an activator, can contribute to the attainment of growth factor autonomy by interference with mitogenic pathways distinct from those involving TR.

(4) *A potential role for v-ErbA at nuclear pore complexes*

A portion of v-ErbA expressed in oocytes was present at the nuclear membrane at the cytoplasmic face of NPC complexes, near the cytoplasmic annuli. Specifically, colloidal gold-labelled v-ErbA was seen at the cytoplasmic fibrils of the NPCs, possibly at fibril components involved in NLS-mediated ligand docking (Forbes, 1992; Görlich *et al.*, 1994). NPCs perform a crucial role in controlling cellular processes. In the yeast *S. cerevisiae*, mutations in NPC proteins can cause nuclear

transport defects, extensive changes in nuclear membrane structure, and abnormal nuclear migration (reviewed in Doye and Hurt, 1995). That components of the import machinery can also interfere with cell cycle regulation is demonstrated by the effects of a mutation in the *S. cerevisiae* NLS receptor, Srp1, that targets proteins to the NPC (Loeb *et al.*, 1995). A conditional *srp1* mutant arrests cells at the G_2/M border, and was suggested to act by blocking the import of a critical cell cycle regulator. Similarly, Pendulin (or *OHO31*), a *Drosphila* tumour suppressor gene product with sequence homology to Srp1, exhibits cell-cycle dependent nuclear localization and is required for normal cell proliferation (Küssel and Frasch, 1995; Török *et al.*, 1995). In the release from G_2 arrest in oocytes, potential effects of v-ErbA at NPCs would be expected to play an early inductive role, as the association of v-ErbA with NPCs occurs well in advance of meiotic events. An isolated local effect of v-ErbA on nuclear membrane structure appears unlikely, since the set of ultrastructural changes induced by v-ErbA, including the observed nuclear membrane alterations, was fully consistent with the pleiomorphic effects of meiotic induction. While the significance of the presence of v-ErbA at NPCs remains to be determined, it is interesting to consider the possibility that, in addition to its intranuclear function in gene activation, v-ErbA might modulate nucleocytoplasmic transport of a key cell cycle regulator(s).

IV.

Conclusion

Until recently, an almost exclusive emphasis on the mutational aspects of oncogenesis, based on retroviral model systems, has tended to relegate the cell as a whole to a secondary background role. Contrasting with this approach, expression of TR and v-ErbA in *Xenopus* oocytes has highlighted the importance of cell-specific molecular interactions in defining the properties of both transcription factors. In Chapter II, it was shown that both the cell-specific nuclear environment and protein-DNA interactions at enhancers determine whether these nuclear factors act as repressors or activators of gene transcription. Importantly, the dominant negative phenotype of v-ErbA was shown not to be universal, but to be dependent on interactions with other nuclear factors and the nature of a given DNA binding site. The importance of these findings for an understanding of growth-promotion by v-ErbA is demonstrated in Chapter III, where it was shown that v-ErbA is able to partially lift the G_2/M cell cycle arrest in fully grown oocytes. v-ErbA induced a subset of early to intermediate meiotic events by activating *de novo* gene expression, suggesting that v-ErbA is able to interfere with mitogenic signalling pathways.

Abbreviations used in this chapter : DR4, direct repeat of TRE half sites separated by 4 nucleotides; LTR, long terminal repeat; NF-κB, nuclear factor-κB; RXR, retinoid X receptor; Sp1, specificity protein 1; TR, thyroid hormone receptor; TRE, thyroid hormone response element.

(1) ***The action of nuclear hormone receptors is dependent on molecular interactions***

The function of nuclear hormone receptors is still understood predominantly in a context-independent way, as being determined by their three-dimensional structures, which are implicit in their primary amino acid sequences; and, at a more reductive level, the functional domains within receptors are seen as autonomous modules. However, recent studies characterizing the modulating roles of protein-protein (Chen and Evans, 1995; Forman *et al.*, 1995b; Hörlein *et al.*, 1995; Kurokawa *et al.*, 1995; Lee *et al.*, 1995a and 1995b) and protein-nucleic acid interactions (Kurokawa *et al.*, 1995; Rastinejad *et al.*, 1995; Sjöberg and Vennström, 1995; Toney *et al.*, 1993), as well as reports on interdomain allosteric regulation (Durand *et al.*, 1994; Kurokawa *et al.*, 1994), indicate that this view inadequately reflects nuclear receptor action. The findings on TR and v-ErbA function reported in my dissertation, together with these studies, demonstrate a pressing need for an expanded conceptual framework on which to base nuclear receptor research. Hence, I propose a context-sensitive model of nuclear receptor action, which views receptor protein functions as plastic properties, requiring definition by molecular interactions.

To allow responsiveness to the complex requirements of development, differentiation and the cell cycle, eukaryotic genes are coupled via cellular signal transduction networks to their extra- and intra-cellular environments. Cancer represents a range of disorders in cellular signal processing, mediated by the abnormal function of regulatory network components. In both healthy and neoplastic cells, the information transmitted to the genome via multiple signal pathways is combined at DNA control sequences by complex assemblages of transcription factors,

and then transmitted to the general transcription machinery. Many different types of signals are processed in this way; for example, information about a cell's lineage, its interactions with other cells, the stage of the cell division cycle, and hormonal signals. Individual regulatory elements function as multifactorial switches, each being responsible for only a certain aspect of the regulation of a given gene. Consequently, the full regulatory scope of a gene is only achieved by the integrated action of all, or of changing sub-sets, of its regulatory elements. This is well illustrated by the action of TR at TR-regulated genes which are commonly controlled by complex patterns of different control elements. The chicken lysozyme gene, for example, is regulated by the combined activity of thyroid and steroid response elements, enhancer sequences and silencer elements, to the effect that an entire chromatin loop with extensive flanking parts on both sides of the transcribed region acts as a regulatory unit (Sippel and Renkawitz, 1989).

Another consequence of the modular composition of complex enhancers is that different combinations of binding sites generate different transcriptional patterns. Modulation of the mode of action of TR/RXR heterodimers by different TREs provides an interesting example. My study showed that mammalian TR/RXR heterodimers constitutively activated transcription from a palindromic TRE linked to a viral promoter in *Xenopus* oocytes. In contrast, *Xenopus* TR/RXR heterodimers act as efficient activators and repressors of the 1.6 kb promoter of the *Xenopus* TRßA gene, containing several TREs within extensive flanking regions (Machuca *et al.*, 1995), in the presence or absence of T_3 respectively (Wong and Shi, 1995). While species-specific interactions between the nuclear receptors and general transcription factors might have contributed to these differences, this comparison underscores the

importance of enhancer composition for the action of TR and RXR. The effects of allosteric interactions between RXR and TR, or RXR and the orphan receptor Nurr1, serve to further illustrate this point (Forman *et al.*, 1995). Heterodimerization between TR and RXR, bound at two consensus half-sites in a DR4 or palindromic arrangement, results in the suppression of ligand-induced transcriptional activities of RXR. Interestingly, without binding directly to DNA, RXR can heterodimerize with a Nurr1 monomer bound to a single half-site. As a result of this interaction, the constitutive activity of Nurr1 is suppressed, and the complex becomes 9-*cis* retinoic acid dependent. As an additional example of binding site complexity, the human immunodeficiency virus type 1 (HIV-1) LTR is activated by TR binding to TREs contained within the viral NF-κB and Sp1 DNA motifs (Desai-Yajnik and Samuels, 1993). At the Sp1 element, the binding of TR and Sp1 is mutually exclusive, and direct interaction between the retroviral *tat* protein and TR is required for TR binding and transcriptional induction of the TRE within the Sp1 motif.

Where oligomeric protein complexes bind to the same control sequence, different combinations of proteins can have different functional activities, including DNA binding specificities (Diamond *et al.*, 1990; Glass, 1994). The consequences can range from efficient activation to complete inhibition, and can change in response to environmental and differentiation signals. Thus, the diversity provided by dimerization, between nuclear hormone receptors (Mangelsdorf and Evans, 1992) and among different nuclear factor families (Karin *et al.*, 1993), offers enormous scope for regulation. In addition, interactions with tissue-specific factors can have profound effects on receptor action. My investigation demonstrated that the v-*erbA* protein, which is generally classified as a dominant negative transcription factor in animal

cells, is converted into a transcriptional activator by factors present in nuclear extracts from anterior pituitary cells. It follows that, if a factor can act as an activator or repressor depending upon its interactions with other proteins, it is the balance of these proteins in the cell and their relative strengths of interaction that is of paramount importance. In conclusion, the combinatorial nature of the transcription process strongly supports a context-sensitive model of nuclear receptor action. Understanding the complexities of how molecular interactions, involving normal and oncogenic nuclear receptors, change in response to environmental, proliferation and differentiation signals will be an exciting focus of future research.

(2) *Signalling networks and cellular architecture are interdependent*

v-ErbA initiated early to intermediate meiotic events in *Xenopus* oocytes by activating *de novo* gene expression. The finding that v-ErbA initiated the release from G_2 arrest in oocytes, but did not induce advanced meiotic events, highlights the strictly cooperative nature of the v-*erbA* oncogene. In somatic cells, v-ErbA depends on the presence of an oncogenic protein kinase, such as v-*erbB* or v-*src*, for the achievement of a fully transformed phenotype (Kahn *et al.*, 1986; Frykberg *et al.*, 1983). These observations emphasize that the effects of the v-*erbA* oncogene are embedded within a complex cellular signalling network made up of mutually interdependent components. Mitogenic signalling cascades constitute sequences of biochemical reactions that directly link signals initiated by receptors at the plasma membrane to specific cytoskeletal and transcriptional targets for control of the cell cycle and differentiation (Davis, 1993; Mordret, 1993). In the nucleus, cytoplasmic signalling cascades are integrated with the hormonal signals conveyed by steroid and

thyroid hormone receptors by complex cross-talk mechanisms (Desbois *et al.*, 1991a, 1991b; Diamond *et al.*, 1990; Jonat *et al.*, 1990; Karin *et al.*, 1993; Schüle *et al.*, 1990a, 1990b; Zhang *et al.*, 1991). Signal amplification along the branching structure of network cascades leads to the activation of increasing numbers of targets at successive levels of signal transduction. Through this mechanism, the action of a single oncogenic receptor kinase at the cell surface, such as v-ErbB, can be sufficient to induce complete cellular transformation; and expression of tyrosine kinase oncogenes, such as *tpr-met* and *RET*, efficiently induces maturation in oocytes (Daar *et al.*, 1991; Grieco *et al.*, 1995). In contrast, the oncogenic transcription factor v-ErbA, acting at the most distal level of mitogenic cascades, may only be able to activate a subset of genes essential for cellular growth-promotion, and, in oocytes, only induces a subset of meiotic events.

The transformation of an oocyte into an egg provides a valuable example for the general requirement of a combination of intracellular signals for the induction of an integrated structural and functional response. Meiosis is orchestrated by subsets of biochemical signal cascades, which independently regulate distinct structural reorganizations, with the ultimate outcome of dramatically altering cell function (Bement and Capco, 1990). Thus, intracellular signalling could be seen as a system which processes the information transmitted by all pathways in a parallel distributed network. Similar models of biological information processing have been proposed to explain such diverse processes as brain function (revewed in Rowe, 1995) and differentiation and development (Atlan and Koppel, 1990). Possibly, these models reflect certain universal principles of signal processing, operating at all organizational levels in biological systems. A cell receives a constant stream of signals both from

within the cell itself and from the surrounding environment, and information is most likely carried by the changing patterns and intensities of all signalling events combined. These patterns are created by the substrate specificity of regulatory kinases in mitogenic cascades, and the binding strengths of protein-protein and protein-nucleic acid interactions in nuclear receptor signalling. Importantly, the cell-specific expression of numerous network components can greatly alter the response to upstream and cross-talk signals.

Cells interpret biological information within three-dimensional networks in which structure and function are mutually dependent. Unfortunately, studies of cell structure have often been judged to be of only observational interest and of lesser scientific value than biochemical approaches. However, the study of nuclear architecture is now moving to the forefront of molecular biology, as its crucial role in gene regulation (see, for example, Blobel, 1985; Nigg, 1988; Bidwell *et al.*, 1993; Cremer *et al.*, 1993; Dworetzky *et al.*, 1992), differentiation, growth control and neoplastic transformation has been recognized (reviewed in Brasch and Ochs, 1995; Penman, 1995; Pienta *et al.*, 1989). It has also been proposed that structural information, derived from cell-cell contacts or cell shape, can be transmitted to the nuclear matrix by the cytoskeleton, and might influence gene transcription (Cook, 1989; Pienta *et al.*, 1989).

Genetic instability, activation of oncogenes and deregulated signal transduction are direct causes of cellular transformation. It is important to remember that virtually every component of the cytoskeleton and the nuclear matrix can also be altered in cancer cells; and that alteration of cell structure is in fact the morphological hallmark of cancer diagnosis (Pienta *et al.*, 1989, and refs. therein). It is therefore necessary to

define the relationship between abnormal signalling events induced by oncogene products, cell structure and genetic changes. The products of oncogenes and their targets can be pictured as sensitive nodes within integrated regulatory-structural networks, whose activity is capable of profoundly altering the global state of the cell. These considerations motivated me to take a combined biochemical and ultrastructural approach in my investigation of v-ErbA-initiated maturation events in *Xenopus* oocytes. M-phase initiation by v-ErbA through gene activation, shown by my study, exemplifies oncogene action through interference with mitogenic signalling networks. In addition, I identified an abnormal subcellular localization for v-ErbA, which was partially found associated with the pore complexes at the cytoplasmic face of the nuclear envelope. This finding poses the intriguing question of whether v-ErbA can also disrupt regulatory networks by interfering with cellular architecture, in particular, components of NPCs. In conclusion, my study as a whole contributes a range of new perspectives on the v-*erbA* oncogene and its action within the regulatory-structural networks of cells.

(3) *Network models of neoplastic cells will open up new avenues in cancer research*

Biological models change with time (Keller, 1995). In the 1950s and 1960s, cellular processes were explained in terms of biochemical pathways and the thermodynamic principles of reaction kinetics. The ability of a cell to establish metabolic pathways was believed to depend solely on the execution of a fixed genetic program (reviewed in Keller, 1995). During this period, studies of two DNA tumour viruses, polyomavirus and simian virus 40, belonging to the papovavirus

group, suggested that the addition of one or a few genes could dramatically alter cell behaviour (reviewed in Eckhart, 1989). This led to the realization that neoplastic changes could be analyzed genetically and biochemically, and marked the beginning of experimental oncology (reviewed in Varmus, 1989). Cancer came to be seen as a genetic disease. Starting from this premise, experimental oncology has succeeded in identifying viral and cellular oncogenes as well as tumour suppressor genes, and has established models of neoplastic transformation possessing extraordinary explanatory power (reviewed in Chapter I). Importantly, models of collaborating viral oncogenes, such as v-*erbA* and v-*erbB*, have led to the multistep hypothesis of carinogenesis (reviewed in Weinberg, 1989b).

In the 1990s, through the discovery of allosteric control, the intricacies of transcriptional regulation and the complexities of the cell cycle, cells are increasingly coming to be seen as intricate networks of structures and information. We now know that genomes do not function as fixed programs, and that cells actively regulate their genes in response to their own internal state (reviewed in McKnight and Yamamoto, 1992) and a wide range of signals from their environment, including those conveyed by hormones (reviewed in Hill and Treisman, 1995). Steroid and thyroid hormones comprise the broadest class of gene regulatory agents known (Brasch and Ochs, 1995; Detera-Wadleigh and Fanning, 1994; Laudet *et al.*, 1992). Their receptors, belonging to the diverse superfamily of nuclear receptors, play a central role in the regulatory networks that transmit signals, originating from the extracellular environment, to the genome through a complex sequence of molecular interactions. Together, these insights have important implications for cancer research. In the United States, for example, hormone-related cancers account for more than

20% of all newly diagnosed male and more than 40% of all newly diagnosed female malignancies (Henderson *et al.*, 1993). Hormonal signals contribute to the causation and progression of several human cancers, including cancer of the breast, endometrium, prostate and ovaries. The interplay between hormonal signals, normal and oncogenic nuclear receptors, and additional activated oncogenes therefore constitutes an area of primary importance in cancer research. Furthermore, oncogene function needs to be understood within the context of the global state of structural and regulatory networks of neoplastic cell types. In the future, the development of network models of neoplastic cells, reflecting these complexities, will expand our understanding of the role of oncogenes in human cancer.

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References

Allan, G. F., X. Leng, S. Y. Tsai, N. L. Weigel, D. P. Edwards, M.-J. Tsai, and B. W. O'Malley. 1992. Hormone and antihormone induce distinct conformational changes which are central to steroid receptor activation. J. Biol. Chem. 267:19513-19520.

Allison, L. A., P. J. Romaniuk, and A. H. Bakken. 1991. RNA-protein interactions of stored 5S RNA with TFIIIA and ribosomal protein L5 during *Xenopus* oogenesis. Dev. Biol. 144:129-144.

Allison, L. A., M. T. North, K. J. Murdoch, P. J. Romaniuk, S. Deschamps, and M. le Maire. 1993. Structural requirements of 5S rRNA for nuclear transport, 7S ribonucleoprotein particle assembly, and 60S ribosomal subunit assembly in *Xenopus* oocytes. Mol. Cell. Biol. 13:6819-6831.

Almouzni, G., and A. P. Wolffe. 1993a. Replication coupled chromatin assembly is required for the repression of basal transcription in vivo. Genes Dev. 7:2033-2047.

Almouzni, G., and A. P. Wolffe. 1993b. Nuclear assembly, structure, and function: the use of Xenopus in vitro systems. Exp. Cell Res. 205:1-15.

Arion, D., L. Meijer, L. Brizuela and D. Beach. 1988. *cdc2* is a component of the M phase-specific histone H1 kinase: evidence for identity with MPF. Cell 55:371-378.

Atlan, H., and M. Koppel. 1990. The cellular computer DNA: program or data. B. Math. Biol. 52:335-348.

Avruch, J., X.-F. Zhang, and J. M. Kyriakis. 1994. Raf meets Ras: completing the framework of a signal transduction pathway. Trends Biochem. Sci. 19:279-283.

Baltus, E., J. Brachet, J. Hanocq-Quertier, and E. Hubert. 1973. Cytochemical and biochemical studies on progesterone-induced maturation in amphibian oocytes. Differentiation 1:127-143.

Banerji, J., S. Rusconi, and W. Schaffner. 1981. Expression of a ß-globin gene is enhanced by remote SV40 DNA sequences. Cell 27:299-308.

Baniahmad, A., C. Steiner, A. K. Kohne, and R. Renkawitz. 1990. Modular structure of a chicken lysozyme silencer: involvement of an unusual thyroid hormone receptor binding site. Cell 61:505-514.

Baniahmad, A., A. K. Kohne, and R. Renkawitz. 1992. A transferable silencing domain is present in the thyroid hormone receptor, in the v-erbA oncogene product and in the retinoic acid receptor. EMBO J. 11:1015-1023.

Baniahmad, A., I. Ha, D. Reinberg, S. Y. Tsai, M.-J. Tsai, and B. W. O'Malley. 1993. Interaction of human thyroid hormone receptor ß with transcription factor TFIIB may mediate target gene derepression and activation by thyroid hormone. Proc. Natl. Acad. Sci. USA 90:8832-8836.

Baniahmad, A., X. Leng, T. P. Burris, S. Y. Tsai, M.-J. Tsai, and B. W. O'Malley. 1995. The τ4 activation domain of the thyroid hormone receptor is required for release of a putative corepressor(s) necessary for transcriptional silencing. Mol. Cell. Biol. 15:76-86.

Banker, D. E., J. Bigler, and R. N. Eisenman. 1991. The thyroid hormone receptor gene (*c-erbA*α) is expressed in advance of thyroid gland maturation during early embryonic development of *Xenopus* laevis. Mol. Cell. Biol. 11:5079-5089.

Barettino, D., M. V. Ruiz, and H. G. Stunnenberg. 1994. Characterization of the ligand-dependent transactivation domain of thyroid hormone receptor. EMBO J. 13:3039-3049.

Barlow, C., B. Meister, M. Lardelli, U. Lendahl, and B. Vennström. 1994. Thyroid abnormalities and hepatocellular carcinoma in mice transgenic for v-*erbA*. EMBO J. 13:4241-4250.

Beato, M. 1989. Gene regulation by steroid hormones. Cell 56:335-344.

Bement, W. M., and D. G. Capco. 1989. Intracellular signals trigger ultrastructural events characteristic of meiotic maturation in oocytes of *Xenopus laevis*. Cell Tissue Res. 255:183-191.

Bement, W. M., and D. G. Capco. 1990. Transformation of the amphibian oocyte into the egg: Structural and biochemical events. J. Electron Microsc. Tech. 16:202-234.

Benavente, R., G. Krohne, and W. W. Franke. 1985. Cell type-specific expression of nuclear lamina proteins during development of Xenopus laevis. Cell 41:177-190.

Beug, H., S. Palmieri, C. Freudenstein, H. Zentgraf and T. Graf. 1982. Hormone-dependent terminal differentiation in vitro of chicken erythroleukemia cells transformed by *ts* mutants of avian erythroblastosis virus. Cell 28:907-919.

Bhat, M. K., C. Parkison, P. McPhie, C.-M. Liang, and S.-Y. Cheng. 1993. Conformational changes of human ß1 thyroid hormone receptor induced by binding of 3,5,3′-triiodo-L-thyronine. Biochem. Biophys. Res. Com. 195:385-392.

Bidwell, J. P., A. J. van Wijnen, E. G. Fey, S. Dworetzky, S. Penman, J. L. Stein, J. B. Lian, and G. S. Stein. 1993. Osteocalcin gene promoter-binding factors are tissue-specific nuclear matrix components. Proc. Natl. Acad. Sci. USA 90:3162-3166.

Bigler, J., and R. N. Eisenman. 1994. Isolation of a thyroid hormone-responsive gene by immunoprecipitation of thyroid hormone receptor-DNA complexes. Mol. Cell. Biol. 14:7621-7632.

Birchmeier, C., D. Broek, and M. Wigler. 1985. RAS proteins can induce meiosis in *Xenopus* oocytes. Cell 43:615-621.

Blobel, G. 1985. Gene gating: A hypothesis. Proc. Natl. Acad. Sci. USA 82:8527-8529.

Bodner, M., J.-L. Castrillo, L. E. Theill, T. Deerinck, M. Ellisman, and M. Karin. 1988. The pituitary-specific transcription factor GHF-1 is a homeobox-containing protein. Cell 55:505-518.

Bogerd, A. M., J. A. Hoffman, D. C. Amberg, G. R. Fink, and L. I. Davis. 1994. *nup1* mutants exhibit pleiotropic defects in nuclear pore complex function. J. Cell Biol. 127:319-332.

Bonde, B. G., M. Sharif, and M. L. Privalsky. 1991. Ontogeny of the *v-erbA* oncoprotein from the thyroid hormone receptor: an alteration in the DNA binding domain plays a crucial role for *v-erbA* function. J. Virol. 65:2037-2046.

Boulikas, T. 1994. Putative nuclear localization signals (NLS) in protein transcription factors. J. Cell. Biochem. 55:32-58.

Brachet, J., F. Hanocq, and P. Van Gansen. 1970. A cytochemical and ultrastructural analysis of *in vitro* maturation in amphibian oocytes. Dev. Biol. 21:157-195.

Brasch, K., and R. L. Ochs. 1995. Nuclear remodeling in response to steroid hormone action. Int. Rev. Cytol. 159:161-194.

Brent, G. A., P. R. Larsen, J. W. Harney, R. J. Koenig, and D. D. Moore. 1989. Functional characterization of the rat growth hormone promoter elements required for induction by thyroid hormone with and without co-transfected ß type thyroid hormone receptor. J. Biol. Chem. 264:178-182.

Brent, G. A., D. D. Moore, and P. R. Larsen. 1991. Thyroid hormone regulation of gene expression. Annu. Rev. Physiol. 53:17-35.

Brinster, R. L., J. M. Allen, R. R. Behringer, R. E. Gelinas, and R. D. Palmiter. 1988. Introns increase transcriptional efficiency in transgenic mice. Proc. Natl. Acad. Sci. USA 85:836-840.

Bugge, T. H., J. Pohl, O. Lonnoy, and H. G. Stunnenberg. 1992. RXRα, a promiscuous partner of retinoic acid and thyroid hormone receptors. EMBO J. 11:1409-1418.

Bukrinsky, M. I., S. Haggerty, M. P. Dempsey, N. Sharova, A. Adzhubei, L. Spitz, P. Lewis, D. Goldfarb, M. Emerman, and M. Stevenson. 1993. A nuclear localization signal within HIV-1 matrix protein that governs infection of non-dividing cells. Nature (London) 365:666-669.

Buratowski, S., and P. A. Sharp. 1992. Initiation of transcription by RNA polymerase II. In McKnight, S.L., and K. R. Yamamoto (eds.) *Transcriptional Regulation.* Monograph 22. New York, Cold Spring Harbor Laboratory Press. p. 227-246.

Burke, B., and L. Gerace. 1986. A cell free system to study reassembly of the nuclear envelope at the end of mitosis. Cell 44:639-652.

Burnside, J., D. S. Darling, and W. W. Chin. 1990. A nuclear factor that enhances binding of thyroid hormone receptors to thyroid hormone response elements. J. Biol. Chem. 265:2500-2504.

Byrd, D. A., D. J. Sweet, N. Pante, K. N. Konstantinov, T. Guan, A. C. S. Saphire, P. J. Mitchell, C. S. Cooper, U. Aebi, and L. Gerace. 1994. Tpr, a large coiled

coil protein whose amino terminus is involved in activation of oncogenic kinases, is localized to the cytoplasmic surface of the nuclear pore complex. J. Cell Biol. 127:1515-1526.

Call, M., T. Glaser, C. Y. Ito, A. J. Buckler, J. Pelletier, D. A. Haber, E. A. Rose, A. Krai, H. Yeger, W. Lewis, C. Jones, and D. Housman. 1990. Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor locus. Cell 60:509-520.

Casanova, J., E. Helmer, S. Selmi-Ruby, J.-S. Qi, M. Au-Fliegner, V. Desai-Yajnik, N. Koudinova, F. Yarm, B. M. Raaka, and H. H. Samuels. 1994. Functional evidence for ligand-dependent dissociation of thyroid hormone and retinoic acid receptors from an inhibitory cellular factor. Mol. Cell. Biol. 14:5756-5765.

Cavenee, W. K., T. P. Dryja, R. A. Phillips, W. F. Benedict, R. Godbout, B. L. Gallie, A. L. Murphree, L. C. Strong, and R. White. 1983. Expression of recessive alleles by chromosomal mechanisms in retinoblastoma. Nature (London) 305:779-784.

Chatterjee, V. K. K., and J. R. Tata. 1992. Thyroid hormone receptors and their role in development. Cancer Surveys 14:147-167.

Chen, H.-W., Z. Smit-McBride, S. Lewis, M. Sharif, and M. L. Privalsky. 1993. Nuclear hormone receptors involved in neoplasia: ErbA exhibits a novel DNA sequence specificity determined by amino acids outside of the zinc finger region. Mol. Cell. Biol. 13:2366-2376.

Chen, H.-W., and M. L. Privalsky. 1993. The *erbA* oncogene represses the actions of both retinoid X and retinoid A receptors but does so by distinct mechanisms. Mol. Cell. Biol. 13:5970-5980.

Chen, J. D., and R. M. Evans. 1995. A transcriptional co-repressor that interacts with nuclear hormone receptors. Nature (London) 377:454-457.

Choi, T., F. Aoki, M. Mori, M. Yamashita, Y. Nagahama, and K. Kohmoto. 1991. Activation of $p34^{cdc2}$ protein kinase activity in meiotic and mitotic cell cycles in mouse oocytes and embryos. Development 113:789-795.

Colman, A. 1984. Expression of exogenous DNA in *Xenopus* oocytes. In Hames, B. D., and S. J. Higgins (eds.) *Transcription and Translation*. Washington DC,

Oxford IRL Press. p. 49-69.

Cook, P. R. 1989. The nucleoskeleton and the topology of transcription. Eur. J. Biochem. 185:487-501.

Cordes, V. C., S. Reidenbach, A. Kohler, N. Stuurman, R. van Driel and W. W. Franke. 1993. Intracellular filaments containing a nuclear pore complex protein. J. Cell Biol. 123:1333-1344.

Cremer, T., A. Kurz, R. Zirbel, S. Dietzel, B. Rinke, E. Schrock, M. R. Speicher, U. Mathieu, A. Jauch, P. Emmerich, H. Scherthan, T. Ried, C. Cremer, and P. Lichter. 1993. Role of chromosome territories in the functional compartmentalization of the cell nucleus. Cold Spring Harbor Symp. Quant. Biol. 58:777-792.

Daar, I. O., G. A. White, S. M. Schuh, D. K. Ferris, and G. F. Vande Woude. 1991. *tpr-met* oncogene product induces maturation-promoting factor activation in *Xenopus* oocytes. Mol. Cell. Biol. 11:5985-5991.

Damm, K., C. C. Thompson, and R. M. Evans. 1989. Protein encoded by v-erbA functions as a thyroid hormone receptor antagonist. Nature (London) 339:593-597.

Damm, K. 1993. ErbA: tumor suppressor turned oncogene? FASEB J. 7:904-909.

Damm, K., and R. M. Evans. 1993. Identification of a domain required for oncogenic activity and transcriptional suppression by v-erbA and thyroid hormone receptor α. Proc. Natl. Acad. Sci. USA 90:10668-10672.

Danielsen, M., L. Hinck, and G. M. Ringold. 1989. Two amino acids within the knuckle of the first zinc finger specify DNA response element activation by the glucocorticoid receptor. Cell 57:1131-1138.

Davis, R. J. 1993. The mitogen-activated protein kinase signal transduction pathway. J. Biol. Chem. 268:14553-14556.

Desai-Yajnik, V., and H. H. Samuels. 1993. The NF-κB and Sp1 motifs of the human immunodeficiency virus type 1 long terminal repeat function as novel thyroid hormone response elements. Mol. Cell. Biol. 13:5057-5069.

Desbois, C., B. Pain, C. Guilhot, M. Benchaibi, M. Ffrench, J. Ghysdael, J-J. Madjar and J. Samarut. 1991a. *v-erbA* oncogene abrogates growth inhibition of chicken embryo fibroblasts induced by retinoic acid. Oncogene 6:2129-2135.

Desbois, C., D. Aubert, C. Legrand, B. Pain and J. Samarut. 1991b. A novel mechanism of action for v-ErbA: Abrogation of the inactivation of transcription factor AP-1 by retinoic acid and thyroid hormone receptors. Cell 67:731-740.

Detera-Wadleigh, S. D., and T. G. Fanning. 1994. Phylogeny of the steroid receptor superfamily. Mol. Phylogenet. Evol. 3:192-205.

Diamond, M. I., J. N. Miner, S. K. Yoshinaga, and K. R. Yamamoto. 1990. Transcription factor interactions: Selectors of positive and negative regulation from a single DNA element. Science 249:1266-1272.

Dingwall, C., and R. A. Laskey. 1991. Nuclear targeting sequences - a consensus? Trends Biochem. Sci. 16:478-481.

Dingwall, C., and R. Laskey. 1992. The nuclear membrane. Science 258:942-947.

Disela, C., C. Glineur, T. Bugge, J. Sap, G. Stengl, J. Dodgson, H. Stunnenberg, H. Beug, and M. Zenke. 1991. v-erbA overexpression is required to extinguish c-erbA function in erythroid cell differentiation and regulation of the erbA target gene CAII. Genes Dev. 5:2033-2047.

Doye, V., and E. C. Hurt. 1995. Genetic approaches to nuclear pore structure and function. Trends Genet. 11:235-241.

Drury, K. C. and S. Schorderet-Slatkine. 1975. Effects of cyclohexamide on the "autocatalytic" nature of the maturation promoting factor (MPF) in oocytes of Xenopus laevis. Cell 4:269-274.

Dumont, J. N. 1972. Oogenesis in *Xenopus laevis* (Daudin) I. Stages of oocyte development in laboratory maintained animals. J. Morphol. 136:153-180.

Dunn, T. M., S. Hahn, S. Ogden, and R. F. Schleif. 1984. An operator at -280 base pairs that is required for repression of *ara*BAD operon promoter: Addition of DNA helical turns between the operator and promoter cyclically hinders repression. Proc. Natl. Acad. Sci. USA 81:5017-5020.

Durand, B., M. Saunders, C. Gaudon, B. Roy, R. Losson, and P. Chambon. 1994. Activation function 2 (AF-2) of retinoic acid receptor and 9-*cis* retinoic acid receptor: presence of a conserved autonomous constitutive activating domain and influence of the nature of the response element on AF-2 activity. EMBO J. 13:5370-5382.

Dworetzky, S. I., K. L. Wright, E. G. Fey, S. Penman, J. B. Lian, J. L. Stein, and G. S. Stein. 1992. Sequence-specific DNA-binding proteins are components of a nuclear matrix-attachment site. Proc. Natl. Acad. Sci. USA 89:4178-4182.

Dynan, W. S. 1989. Modularity in promoters and enhancers. Cell 58:1-4.

Eckhart, W. 1989. Oncogenes of DNA tumor viruses: Papovaviruses. In Weinberg, R. A. (ed.) *Oncogenes and the Molecular Origins of Cancer.* Monograph 18. New York, Cold Spring Harbor Press. p. 223-238.

Eisenman, R. N. 1989 Nuclear oncogenes. In Weinberg, R. A. (ed.) *Oncogenes and the Molecular Origins of Cancer.* Monograph 18. New York, Cold Spring Harbor Press. p. 175-221.

Eliceiri, B. P., and D. D. Brown. 1994. Quantitation of endogenous thyroid hormone receptors α and ß during embryogenesis and metamorphosis in *Xenopus laevis*. J. Biol. Chem. 269:24459-24465.

Evans, R. M. 1988. The steroid and thyroid hormone receptor superfamily. Science 240:889-895.

Fabian, J. R., D. K. Morrison, and I. O. Daar. 1993. Requirement for Raf and MAP kinase function during the meiotic maturation of *Xenopus* oocytes. J. Cell Biol. 122:645-652.

Feinberg, A. P. 1994. A developmental context for multiple genetic alterations in Wilms' tumor. J. Cell Science S18:7-12.

Flug, F., R. P. Copp, J. Casanova, Z. Horowitz, L. Janocko, M. Plotnick, and H. H. Samuels. 1987. *Cis*-acting elements of the rat growth hormone gene which mediate basal and regulated expression by thyroid hormone. J. Biol. Chem. 262:6373-6382.

Fondell, J. D., A. L. Roy, and R. G. Roeder. 1993. Unliganded thyroid hormone receptor inhibits formation of a functional preinitiation complex: implications for active repression. Genes Dev. 7:1400-1410.

Forbes, D. J. 1992. Structure and function of the nuclear pore complex. Ann. Rev. Cell Biol. 8:495-527.

Forman, B. M., C.-R. Yang, F. Stanley, J. Casanova, and H. H. Samuels. 1988. c-erbA protooncogenes mediate thyroid hormone-dependent and independent regulation of the rat growth hormone and prolactin genes. Mol. Endocrinol.

2:902-911.

Forman, B. M., and H. H. Samuels. 1990. Interactions among a subfamily of nuclear hormone receptors: the regulatory zipper model. Mol. Endocrinol. 4:1293-1301.

Forman, B. M., J. Casanova, B. M. Raaka, J. Ghysdael, and H. H. Samuels. 1992. Half-site spacing and orientation determines whether thyroid hormone and retinoic acid receptors and related factors bind to DNA response elements as monomers, homodimers, or heterodimers. Mol. Endocrinol. 6:429-442.

Forman, B. M., E. Goode, J. Chen, A. E. Oro, D. J. Bradley, T. Perlman, D. J. Noonan, L. T. Burka, T. McMorris, W. W. Lamph, R. M. Evans, and C. Weinberger. 1995a. Identification of a nuclear receptor that is activated by farnesol metabolites. Cell 81:687-693.

Forman, B. M., K. Umesono, J. Chen, and R. M. Evans. 1995b. Unique response pathways are established by allosteric interactions among nuclear hormone receptors. Cell 81:541-550.

Fornerod, M., J. Boer, S. van Baal, M. Jaegle, M. von Lindern, K. G. Murti, D. Davis, J. Bonten, A. Buijs, and G. Grosveld. 1995. Relocation of the carboxyterminal part of CAN from the nuclear envelope to the nucleus as a result of leukemia-specific chromosome rearrangements. Oncogene 10: 1739-1748.

Forrest, D., A. Munoz, C. Raynoschek, B. Vennström, and H. Beug. 1990. Requirement for the C-terminal domain of the *v-erbA* oncogene protein for biological function and transcriptional repression. Oncogene 5:309-316.

Fox, S. R., M. T. C. Jong, J. Casanova, Z.-S. Ye, F. Stanley, and H. H. Samuels. 1990. The homeodomain protein, Pit-1/GHF-1, is capable of binding to and activating cell-specific elements of both the growth hormone and prolactin gene promoters. Mol. Endocrinol. 4:1069-1080.

Frawley, L. S. 1989. Mammosomatotropes: current status and possible functions. Trends Endocrinol. Metab. 1:31-34.

Freake, H. C., A. Santos, Y. Goldberg, J. Ghysdael, and J. H. Oppenheimer. 1988. Differences in antibody recognition of the triiodothyronine nuclear receptor and c-erbA products. Mol. Endocrinol. 2:986-991.

Freedman, L. P., B. F. Luisi, Z. R. Korszun, R. Basavappa, P. B. Sigler, and K. R. Yamamoto. 1988. The function and structure of the metal coordination sites within the glucocorticoid receptor DNA binding domain. Nature (London) 334:543-546.

Freeman, R. S., J. P. Kanki, S. M. Ballantyne, K. M. Pickham, and D. J. Donoghue. 1990. Effects of the v-*mos* oncogene on *Xenopus* development: meiotic induction in oocytes and mitotic arrest in cleaving embryos. J. Cell Biol. 111:533-541.

Fry, A. M., and E. A. Nigg. 1995. The NIMA kinase joins forces with Cdc2. Curr. Opin. Cell Biol. 5:1122-1125.

Frykberg, L., S. Palmieri, H. Beug, T. Graf, M. J. Hayman and B. Vennström. 1983. Transforming capacities of avian erythroblastosis virus mutants deleted in the *erbA* or *erbB* oncogenes. Cell 32:227-238.

Fuerstenberg, S., I. Leitner, C. Schroeder, H. Schwarz, B. Vennström, and H. Beug. 1992. Transcriptional repression of band 3 and CAII in *v-erbA* transformed erythroblasts accounts for an important part of the leukaemic phenotype. EMBO J. 11:3355-3365.

Gandrillon, O., P. Jurdic, M. Benchaibi, J.-H. Xiao, J. Ghysdael, and J. Samarut. 1987. Expression of the v-erbA oncogene in chicken embryo fibroblasts stimulates their proliferation in vitro and enhances tumor growth in vivo. Cell 49:687-697.

Gandrillon, O., N. Ferrand, J-J. Michaille, L. Roze, M. H. Zile and J. Samarut. 1994. c-*erbA* α/T3R and RARs control commitment of hematopoietic self-renewing progenitor cells to apoptosis or differentiation and are antagonized by the v-*erbA* oncogene. Oncogene 9:749-758.

Gavin, A.- C., J. D. Vassalli, J.-C. Cavadore, and S. Schorderet-Slatkine. 1992. Okadaic acid and $p13^{suc1}$ modulate the reinitiation of meiosis in mouse oocytes. Mol. Reprod. Rev. 33:287-296.

Gavin, A-C., J.-C. Cavadore, and S. Schorderet-Slatkine. 1994. Histone H1 kinase activity, germinal vesicle breakdown and M phase entry in mouse oocytes. J. Cell Sci. 107:275-283.

Gerace, L., and G. Blobel. 1980. The nuclear envelope lamina is reversibly depolymerized during mitosis. Cell 19:277-287.

Gerace, L. 1992. Molecular trafficking across the nuclear pore complex. Curr. Opin. Cell Biol. 4:637-645.

Gerace, L., and R. Foisner. 1994. Integral membrane proteins and dynamic organization of the nuclear envelope. Trends Cell Biol. 4:129-131.

Gerhart, J., M. Wu, and M. Kirschner. 1984. Cell cycle dynamics of an M-phase-specific cytoplasmic factor in *Xenopus laevis* oocytes. J. Cell Biol. 98:1247-1255.

Gessler, M., A. Poustka, W. Cavenee, R. L. Neve, S. H. Orkin, and G. A. P. Bruns. 1990. Homozygous deletion in Wilms' tumours of a zinc-finger gene identified by chromosome jumping. Nature (London) 343:774-778.

Glass, C. K., J. M. Holloway, O. V. Devary, and M. G. Rosenfeld. 1988. The thyroid hormone receptor binds with opposite transcriptional effects to a common sequence motif in thyroid hormone and estrogen response elements. Cell 54:313-323.

Glass, C. K., and M. G. Rosenfeld. 1991. Regulation of gene transcription by thyroid hormones and retinoic acid. In Foulkes, G., and P. Cohen (eds.) *The Hormonal Control of Gene Transcription*. Vol.6. Amsterdam, Elsevier. p.299-327.

Glass, C. K. 1994. Differential recognition of target genes by nuclear receptor monomers, dimers, and heterodimers. Endocrinol. Rev. 15:391-407.

Gojobori, T., and S. Yokoyama. 1985. Rates of evolution of the retroviral oncogene of Moloney murine sarcoma virus and of its cellular homologues. Proc. Natl. Acad. Sci. USA 82:4198-4201.

Goldberg, M. W., and T. D. Allen. 1992. High resolution scanning electron microscopy of the nuclear envelope: the baskets of the nucleoplasmic face of the nuclear pores. J. Cell Biol. 119:1429-1440.

Goldberg, M. W., and T. D. Allen. 1995. Structural and functional organization of the nuclear envelope. Curr. Opin. Cell Biol. 7:301-309.

Görlich, D., S. Prehn, R. A. Laskey, and E. Hartmann. 1994. Isolation of a protein that is essential for the first step of nuclear protein import. Cell 79:767-778.

Gorman, C., R. Padmanabhan, and B. H. Howard. 1983. High efficiency DNA-mediated transformation of primate cells. Science 221:551-553.

Gotoh, Y., E. Nishida, S. Matsuda, N. Shiina, H. Kosato, K. Shirokawa, T. Akiyama, K. Ohta, and H. Sakai. 1991. *In vitro* effects on microtubule dynamics of purified *Xenopus* M-phase-activated MAP kinase. Nature (London) 349:251-254.

Graf, T., B. Royer-Pokora, and H. Beug. 1976. Evidence for the multiple oncogenic potential of cloned leukemia viruses: *In vitro* and *in vivo* studies with avian erythroblastosis virus. Virology 71:423-433.

Graf, T., and H. Beug. 1978. Avian leukemia viruses: Interaction with their target cells *in vivo* and *in vitro*. Biochim. Biophys. Acta 516:269-299.

Graf, T., and H. Beug. 1983. Role of the v-*erbA* and v-*erbB* oncogenes of avian erythroblastosis virus in erythroid cell transformation. Cell 34:7-9.

Graziani, G., A. R. Nebreda, S. Srivastava, E. Santos, and A. Eva. 1992. Induction of *Xenopus* oocyte meiotic maturation by the *dbl* oncogene product. Oncogene 7:229-235.

Green, S., and P. Chambon. 1986. A superfamily of potentially oncogenic hormone receptors. Nature (London) 324:615-617.

Grieco, D., M. Santoro, N. A. Dathan, and A. Fusco. 1995. Activated *RET* oncogene products induce maturation of *Xenopus* oocytes. Oncogene 11:113-117.

Gronemeyer, H. 1992. Control of transcription activation by steroid hormone receptors. FASEB J. 6:2524-2529.

Gronemeyer, H., and D. Moras. 1995. How to finger DNA. Nature (London) 375:190-191.

Guiochon-Mantel, A., and E. Milgrom. 1993. Cytoplasmic-nuclear trafficking of steroid hormone receptors. TEM 4:322-328.

Gurdon, J. B., and D. A. Melton. 1981. Gene transfer in amphibian eggs and oocytes. Ann. Rev. Genet. 15:189-218.

Hadzic, E., V. Desai-Yajnik, E. Helmer, S. Guo, S. Wu, N. Koudinova, J. Casanova, B. M. Raaka, and H. H. Samuels. 1995. A 10-amino-acid sequence in the N-terminal A/B domain of thyroid hormone receptor α is essential for transcriptional activation and interaction with the general transcription factor

TFIIB. Mol. Cell. Biol. 15:4507-4517.

Hard, T., E. Kellenbach, R. Boelens, R. Kaptein, K. Dahlman, J. Carlstedt-Duke, L. P. Freedman, B. A. Maler, E. I. Hyde, J. A. Gustafsson, and K. R. Yamamoto. 1990a. ^{1}H NMR studies of the glucocorticoid receptor DNA-binding domain: sequential assignments and identification of secondary structure elements. Biochemistry 29:9015-9023.

Hard, T., E. Kellenbach, R. Boelens, B. A. Maler, K. Dahlman, L. P. Freedman, J. Carlstedt-Duke, K. R. Yamamoto, and R. Kaptein. 1990b. Solution structure of the glucocorticoid receptor DNA-binding domain. Science 249:157-160.

Hattori, S., M. Fukuda, T. Yamashita, S. Nakamura, Y. Gotoh, and E. Nishida. 1992. Activation of mitogen-activated protein kinase and its activator by Ras in intact cells and in a cell-free system. J. Biol. Chem. 267:20346-20351.

Hausen P., and M. Riebesell. 1991. *The Early Development of Xenopus laevis: An Atlas of Histology*. New York, Springer Verlag. 142 pp.

Heikkila, J. J. 1990. Expression of cloned genes and translation of messenger RNA in microinjected *Xenopus* oocytes. Int. J. Biochem. 22:1223-1228.

Henderson, B. E., R. K. Ross, and M. C. Pike. 1993. Hormonal chemoprevention of cancer in women. Science 259:633-638.

Hermann, T., B. Hoffman, F. J. Piedrafita, X.-H. Zhang, and M. Pfahl. 1993. *v-erbA* requires auxiliary protein for dominant negative activity. Oncogene 8: 55-65.

Herskowitz, I. 1987. Functional inactivation of genes by dominant negative mutations. Nature (London) 329:219-222.

Heyman, R. A., D. J. Mangelsdorf, J. A. Dyck, R. B. Stein, G. Eichele, R. M. Evans, and C. Thaller. 1992. 9-cis retinoic acid is a high affinity ligand for retinoid X receptor. Cell 68:397-406.

Hill, C. S., and R. Treisman. 1995. Transcriptional regulation by extracellular signals: Mechanisms and specificity. Cell 80:199-211.

Hinshaw, J. E., B. O. Carragher, and R. A. Milligan. 1992. Architecture and design of the nuclear pore complex. Cell 69:1133-1141.

Hinshaw, J. E. 1994. Architecture of the nuclear pore complex and its involvement in nucleocytoplasmic transport. Biochem. Pharmacol. 47:15-20.

Hollenberg, A. N., T. Monden, and F. E. Wondisford. 1995. Ligand-independent and -dependent functions of thyroid hormone receptor isoforms depend upon their distinct amino termini. J. Biol. Chem. 270:14274-14280.

Holloway, J. M., C. K. Glass, S. Adler, C. A. Nelson, and M. G. Rosenfeld. 1990. The C'-terminal interaction domain of the thyroid hormone receptor confers the ability of the DNA site to dictate positive or negative transcriptional activity. Proc. Natl. Acad. Sci. USA 87:8160-8164.

Hori, R., S. Pyo, and M. Carey. 1995. Protease footprinting reveals a surface on transcription factor TFIIB that serves as an interface for activators and coactivators. Proc. Natl. Acad. Sci. USA 92:6047-6051.

Hörlein, A. J., A. M. Näär, T. Heinzel, J. Torchia, B. Gloss, R. Kurokawa, A. Ryan, Y. Kamei, M. Söderström, C. K. Glass, and M. G. Rosenfeld. 1995. Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. Nature (London) 377:397-403.

Hunter, T. 1989. Oncogene products in the cytoplasm: The protein kinases. In Weinberg, R. A. (ed.) *Oncogenes and the Molecular Origins of Cancer.* Monograph 18. New York, Cold Spring Harbor Press. p. 147-173.

Hurt, E. C. 1993. The nuclear pore complex. FEBS Lett. 325:76-80.

Iglesias, T., S. Llanos, M. Lopez-Barahona, B. Seliger, A. Rodriguez-Pena, J. Bernal, and A. Munoz. 1995. Induction of platelet-derived growth factor B/c-*sis* by the v-*erbA* oncogene in glial cells. Oncogene 10:1103-1110.

Ikeda, M., M. Rhee, and W. M. Chin. 1994. Thyroid hormone receptor monomer, homodimer, and heterodimer (with retinoid-X receptor) contact different nucleotide sequences in thyroid hormone response elements. Endocrinology 135:1628-1637.

Jarnik, M., and U. Aebi. 1991. Toward a more complete 3-D structure of the nuclear pore complex. J. Struct. Biol. 107:291-308.

Jessus, C., C. Thibier, and R. Ozon. 1987. Levels of microtubules during the meiotic maturation of the *Xenopus* oocyte. J. Cell Sci. 87:705-712.

Johnson, A. D. 1995. The price of repression. Cell 81:655-658.

Jonat, C., H. J. Rahmsdorf, K.-K. Park, A. C. B. Cato, S. Gebel, H. Ponta, and P. Herrlich. 1990. Antitumor promotin and antiinflamation: Down-modulation of

AP-1 (fos/jun) activity by glucocorticoid hormone. Cell 62:1189-1204.

Kahn, P., C. Frykberg, C. Brady, I. Stanley, H. Berg, B. Vennström, and T. Graf. 1986. v-*erbA* cooperates with sarcoma oncogenes in leukemic cell transformation. Cell 45:349-356.

Karin, M., H.-F. Yang-Yen, J.-C. Chambard, T. Deng, and F. Saatcioglu. 1993. Various modes of gene regulation by nuclear receptors for steroid and thyroid hormones. Eur. J. Clin. Pharmacol. S45:9-15.

Kato, S., H. Endoh, Y. Masuhiro, T. Kitamoto, S. Uchiyama, H. Sasaki, S. Masushige, Y. Gotoh, E. Nishida, H. Kawashima, D. Metzger, and P. Chambon. 1995. Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. Science 270:1491-1494.

Katz, R., and R. Koenig. 1993. Nonbiased identification of DNA sequences that bind thyroid hormone receptor alpha1 with high affinity. J. Biol. Chem. 268:19392-19397.

Kawahara, A., B. S. Baker, and J. R. Tata. 1991. Developmental and regional expression of thyroid hormone receptor genes during *Xenopus* development. Development 112:933-943.

Keller, E. F. 1995. *Refiguring Life: Metaphors of Twentieth-Century Biology*. New York, Columbia University Press. 134 pp.

Kessel, R. G., and S. Subtelny. 1981. Alteration of annulate lamellae in the in vitro progesterone-treated, full-grown *Rana pipiens* oocyte. J. Exp. Zool. 217:119-135.

Kessel, R. G. 1992. Annulate lamellae: A last frontier in cellular organelles. Int. Rev. Cytol. 133:43-120.

Kliewer, S. A., K. Umesono, D. J. Mangelsdorf, and R. M. Evans. 1992. Retinoid X receptor interacts with nuclear receptors in retinoic acid, thyroid hormone and vitamin D3 signalling. Nature (London) 355:446-449.

Knudson, A. G. 1971. Mutation and cancer: Statistical study of retinoblastoma. Proc. Natl. Acad. Sci. USA 68:820-823.

Kobayashi, H., J. Minshull, C. Ford, R. Golsteyn, R. Poon, and T. Hunt. 1991. On the synthesis and destruction of A- and B-type cyclins during oogenesis and meiotic maturation in *Xenopus laevis*. J. Cell Biol. 114:755-765.

Kosako, H., Y. Gotoh, and E. Nishida. 1994a. MAP kinase cascade in *Xenopus* oocytes. J. Cell Science S18:115-119.

Kosako, H., Y. Gotoh, and E. Nishida. 1994b. Requirement for the MAP kinase kinase/MAP kinase cascade in *Xenopus* oocyte maturation. EMBO J. 13:2131-2138.

Kraemer, D., R. W. Wozniak, G. Blobel, and A. Radu. 1994. The human CAN protein, a putative oncogene product associated with myeloid leukemogenesis, is a nuclear pore complex protein that faces the cytoplasm. Proc. Natl. Acad. Sci. USA 91:1519-1523.

Krohne, G., and W. W. Franke. 1983. Proteins of pore complex-lamina structures from nuclei and nuclear membranes. Methods Enzymol. 96:597-608.

Kurokawa, R., J. Direnzo, M. Boehm, J. Sugarman, B. Gloss, M. G. Rosenfeld, R. A. Heyman, and C. K. Glass. 1994. Regulation of retinoid signalling by receptor polarity and allosteric control of ligand binding. Nature (London) 371:528-531.

Kurokawa, R., M. Söderström, A. Hörlein, S. Halachmi, M. Brown, M. G. Rosenfeld, and C. K. Glass. 1995. Polarity-specific activities of retinoic acid receptors determined by a co-repressor. Nature (London) 377:451-454.

Küssel, P., and M. Frasch. 1995. Pendulin, a *Drosophila* protein with cell cycle-dependent nuclear localization, is required for normal cell proliferation. J. Cell Biol. 129:1491-1507.

LaCasse, E. C., and Y. A. Lefebvre. 1995. Nuclear localization signals overlap DNA- or RNA-binding domains in nucleic acid-binding proteins. Nucleic Acids Res. 23:1647-1656.

Langan, T. A., J. Gautier, M. Lohka, R. Hollingsworth, S. Moreno, P. Nurse, J. Maller and R. A. Sclafani. 1989. Mammalian growth-associated histone H1 kinase: A homolog of *cdc2*$^{+}$*/CDC28* protein kinase controlling mitotic entry in yeast and frog cells. Mol. Cell. Biol. 9:3860-3868.

Laudet, V., C. Hanni, J. Coll, F. Catzeflis, and D. Stehelin. 1992. Evolution of the nuclear receptor gene superfamily. EMBO J. 11:1003-1013.

Lazar, M. A., T. J. Berrodin, and H. P. Harding. 1991. Differential DNA binding by monomeric, homomeric, and potentially heteromeric forms of the thyroid

hormone receptor. Mol. Cell. Biol. 11:5005-5015.

Lee, I. J., P. H. Driggers, J. A. Medin, V. M. Nikodem, and K. Ozato. 1994a. Recombinant thyroid hormone receptor and retinoid X receptor stimulate ligand-dependent transcription *in vitro*. Proc. Natl. Acad. Sci. USA 91:1647-1651.

Lee, J. W., D. D. Moore, and R. A. Heyman. 1994b. A chimeric thyroid hormone receptor constitutively bound to DNA requires retinoid X receptor for hormone-dependent transcriptional activation in yeast. Mol. Endocrinol. 8:1245-1252.

Lee, J. W., H.-S. Choi, J. Gyuris, R. Brent, and D. D. Moore. 1995a. Two classes of proteins dependent on either the presence or absence of thyroid hormone for interaction with the thyroid hormone receptor. Mol. Endocrinol. 9:243-254.

Lee, J. W., F. Ryan, J. C. Swaffield, S. A. Johnston, and D. D. Moore. 1995b. Interaction of the thyroid-hormone receptor with a conserved transcriptional mediator. Nature (London) 374:91-94.

Lee, M. S., S. A. Kliewer, J. Provencal, P. E. Wright, and R. M. Evans. 1993. Solution structure of the RXR DNA-binding protein: a helix required for homodimeric DNA binding. Science 260:1117-1121.

Lefevre, C., M. Imagawa, S. Dana, J. Grindlay, M. Bodner, and M. Karin. 1987. Tissue-specific expression of the human growth hormone gene is conferred in part by the binding of a specific *trans*-acting factor. EMBO J. 6:971-981.

Leid, M., P. Kastner, and P. Chambon. 1992a. Multiplicity generates diversity in the retinoic acid signalling pathways. Trends Biochem. Sci. 17:427-433.

Leid, M., P. Kastner, R. Lyons, H. Nakshatri, M. Saunders, T. Zacharewski, J.-Y. Chen, A. Staub, J.-M. Garnier, S. Mader, and P. Chambon. 1992b. Purification, cloning, and RXR identity of the HeLa cell factor with which RAR or TR heterodimerizes to bind target sequences efficiently. Cell 68:377-395.

Leng, X., S. Y. Tsai, B. W. O'Malley, and M.-J. Tsai. 1993. Ligand-dependent conformational changes in thyroid hormone and retinoic acid receptors are potentially enhanced by heterodimerization with retinoic X receptor. J. Steroid Biochem. Molec. Biol. 46:643-661.

Lessman, C. A. 1987. Germinal vesicle migration and dissolution in Rana pipiens oocytes: effects of steroids and microtubule poisons. Cell Differ. 20:239-251.

Lessman, C. A., and R. G. Kessel. 1992. Effects of acrylamide on germinal vesicle migration and dissolution in oocytes of the frog, *Rana pipiens*. Exp. Cell. Res. 202:151-160.

Levine, M., and J. L. Manley. 1989. Transcriptional repression of eukaryotic promoters. Cell 59:405-408.

Liang, S., M. Hitomi, and A. M. Tartakoff. 1995. Adenoviral E1B-55kDa protein inhibits yeast mRNA export and perturbs nuclear structure. Proc. Natl. Acad. Sci. USA 92:7372-7375.

Loeb, J. D. J., G. Schlenstedt, D. Pellman, D. Kornitzer, P. A. Silver, and G. R. Fink. 1995. The yeast nuclear import receptor is required for mitosis. Proc. Natl. Acad. Sci. USA 92: 7647-7651.

Lohka, M. J., and Y. Masui. 1983. Formation in vitro of sperm pronuclei and mitotic chromosomes induced by amphibian ooplasmic components. Science 220:719-721.

Lohka, M. J., and J. L. Maller. 1985. Induction of nuclear envelope breakdown, chromosome condensation, and spindle formation in cell-free extracts. J. Cell Biol. 101:518-523.

Lu, K. P., and T. Hunter. 1995. Evidence for a NIMA-like mitotic pathway in vertebrate cells. Cell 81:413-424.

Luisi, B. F., W. X. Xu, Z. Otwinowshi, L. P. Freedman, K. R. Yamamoto, and B. Sigler. 1991. Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. Nature (London) 352:497-505.

Macaulay, C., E. Meier, and D. J. Forbes. 1995. Differential mitotic phosphorylation of proteins of the nuclear pore complex. J. Biol. Chem. 270:254-262.

Machuca, I., G. Esslemont, L. Fairclough, and J. R. Tata. 1995. Analysis of structure and expression of the *Xenopus* thyroid hormone receptor-ß gene to explain its autoinduction. Mol. Endocrinol. 9:96-107.

Mader, S., V. Kumar, H. de Verneuil, and P. Chambon. 1989. Three amino acids of the oestrogen receptor are essential to its ability to distinguish an oestrogen from a glucocorticoid response element. Nature (London) 338:271-274.

Mader, S., P. Leroy, J.-Y. Chen, and P. Chambon. 1993. Multiple parameters control the selectivity of nuclear receptors for their response elements: selectivity and promiscuity in response element recognition by retinoic acid receptors and retinoid X receptors. J. Biol. Chem. 268:591-600.

Maldonado, E., and D. Reinberg. 1995. News on initiation and elongation of transcription by RNA polymerase II. Curr. Opin. Cell Biol. 7:352-361.

Maller, J. L. 1990. *Xenopus* oocytes and the biochemistry of cell division. Biochemistry 29:3157-3166.

Mangelsdorf, D. J., and R. M. Evans. 1992. Retinoid receptors as transcription factors. In McKnight, S. L., and K. R. Yamamoto (eds.) *Transcriptional Regulation*. Monograph 22. New York, Cold Spring Harbor Laboratory Press. p.1137-1167.

Mansour, S. J., W. T. Matten, A. S. Hermann, J. M. Candia, S. Rong, K. Fukasawa, G. F. Vande Woude, and N. G. Ahn. 1994. Transformation of mammalian cells by constitutively active MAP kinase kinase. Science 265:966-970.

Marks, M. S., P. L. Hallenbeck, T. Nagata, J. H. Segars, E. Appella, V. M. Nikodem, and K. Ozato. 1992. H-2RIIBP. (RXRß) heterodimerization provides a mechanism for combinatorial diversity in the regulation of retinoic acid and thyroid hormone responsive genes. EMBO J. 11:1419-1435.

Matsuda, S., H. Kosako, K. Takenaka, K. Moriyama, H. Sakai, T. Akiyama, Y. Gotoh, and E. Nishida. 1992. *Xenopus* MAP kinase activator: identification and function as a key intermediate in the phosphorylation cascade. EMBO J. 11:973-982.

Matten, W., I. Daar, and G. F. Vande Woude. 1994. Protein kinase A acts at multiple points to inhibit *Xenopus* oocyte maturation. Mol. Cell. Biol. 14:4419-4426.

Maxson, R., M. Ito, S. Balcells, M. Thayer, M. French, F. Lee, and L. Etkin. 1988. Differential stimulation of sea urchin early and late H2B histone gene expression by a gastrula nuclear extract after injection into *Xenopus laevis* oocytes. Mol. Cell. Biol. 8:1236-1246.

McCormick, F. 1989. *ras* oncogenes. In Weinberg, R. A. (ed.) *Oncogenes and the Molecular Origins of Cancer*. Monograph 18. New York, Cold Spring Harbor Press. p. 125-145.

McKenzie, E. A., N. A. Cridland, and J. Knowland. 1990. Activation of chromosomal vitellogenin genes in *Xenopus* oocytes by pure estrogen receptor and independent activation of albumin genes. Mol. Cell. Biol. 10:6674-6682.

McKnight, S. L., and K. R. Yamamoto (eds.). 1992. *Transcriptional Regulation*. Vol. 1 and 2. Monograph 22. New York, Cold Spring Harbor Laboratory Press. 1334 pp.

Melchior, F., and L. Gerace. 1995. Mechanisms of nuclear protein import. Curr. Opin. Cell Biol. 7:310-318.

Miake-Lye, R., and M. W. Kirschner. 1985. Induction of early mitotic events in a cell-free system. Cell 41:165-175.

Minshull, J., A. Murray, A. Colman, and T. Hunt. 1991. *Xenopus* oocyte maturation does not require new cyclin synthesis. J. Cell Biol. 114:767-772.

Mordret, G. 1993. MAP kinase kinase: A node connecting multiple pathways. Biol. Cell 79:193-207.

Moreau, P., R. Hen, B. Wasylyk, R. Everett, M. P. Gaub and P. Chambon. 1981. The SV40 72 base pair repeat has a striking effect on gene expression both in SV40 and other chimeric recombinants. Nucl. Acids Res. 9:6047-6068.

Mouzaki, A., R. Weil, L. Muster, and D. Rungger. 1991. Silencing and *trans*-activation of the mouse IL-2 gene in *Xenopus* oocytes by proteins from resting and mitogen-induced primary T-lymphocytes. EMBO J. 10:1399-1406.

Munoz, A., M. Zenke, U. Gehring, J. Sap, H. Beug and B. Vennström. 1988. Characterization of the hormone-binding domain of the chicken *c-erbA*/thyroid hormone receptor protein. EMBO J. 7:155-159.

Muramatsu, M.-A., K. Kaibuchi, and K.-I. Arai. 1989. A protein kinase C cDNA without the regulatory domain is active after transfection in vivo in the absence of phorbol ester. Mol. Cell. Biol. 9:831-836.

Murray, M. B., and H. C. Towle. 1989. Identification of nuclear factors that enhance binding of the thyroid hormone receptor to a thyroid hormone response element. Mol. Endocrinol. 3:1434-1442.

Muslin, A. J., A. Klippel, and L. T. Williams. 1993*a*. Phosphatidylinositol 3-kinase activity is important for progesterone-induced *Xenopus* oocyte maturation. Mol. Cell. Biol. 13:6661-6666.

Muslin, A. J., A. M. MacNicol, and L. T. Williams. 1993*b*. Raf-1 protein kinase is important for progesterone-induced *Xenopus* oocyte maturation and acts downstream of mos. Mol. Cell. Biol. 13:4197-4202.

Näär, A. M., J.-M. Boutin, S. M. Lipkin, V. C. Yu, J. M. Holloway, C. K. Glass, and M. G. Rosenfeld. 1991. The orientation and spacing of core DNA-binding motifs dictate selective transcriptional responses to three nuclear receptors. Cell 65:1267-1299.

Nagl, S. B., C. C. Nelson, P. J. Romaniuk, and L. A. Allison. 1995. Constitutive transactivation by the thyroid hormone receptor and a novel pattern of activity of its oncogenic homolog *v-ErbA* in *Xenopus* oocytes. Mol. Endocrinol. 9:1522-1532.

Narasimhan, V., O. Hamill, and R. A. Cerione. 1992. The effects of the normal and oncogenic forms of the neu tyrosine kinase, and the corresponding forms of an immunoglobulin E receptor/neu tyrosine kinase fusion protein, on *Xenopus* oocyte maturation. FEBS Lett. 303:164-168.

Nebreda, A. R., A. Porras, and E. Santos. 1993. $p21^{ras}$-induced meiotic maturation of *Xenopus* oocytes in the absence of protein synthesis: MPF activation is preceded by activation of MAP and S6 kinases. Oncogene 8:467-477.

Nelson, C. C., J. S. Faris, S. C. Hendy, and P. J. Romaniuk. 1993. Functional analysis of the amino acids in the DNA recognition α-helix of the human thyroid hormone receptor. Mol. Endocrinol. 7: 1185-1195.

Newmeyer, D. D., D. R. Finlay, and D. J. Forbes. 1986a. In vitro transport of a fluorescent nuclear protein and exclusion of non-nuclear proteins. J. Cell Biol. 103:2091-2092.

Newmeyer, D. D., J. M. Lucocq, T. R. Burglin, and E. M. De Robertis. 1986b. Assembly *in vitro* of nuclei active in nuclear protein transport: ATP is required for nucleoplasmin accumulation. EMBO J. 5: 501-510.

Newmeyer, D. D., and D. J. Forbes. 1988. Nuclear import can be separated into distinct steps in vitro: nuclear pore binding and translocation. Cell 52:641-653.

Newport, J. W. 1987. Nuclear reconstitution in vitro: stages of assembly around protein-free DNA. Cell 48:205-217.

Newport, J. W., and T. Spann. 1987. Disassembly of the nucleus in mitotic extracts: Membrane vesicularization, lamin disassembly, and chromosome condensation are independent processes. Cell 48: 219-230.

Nigg, E. A. 1988. Nuclear function and organization: the potential of immunochemical approaches. Int. Rev. Cytol. 110:27-92.

Norman, M. F., T. N. Lavin, J. D. Baxter, and B. L. West. 1989. The rat growth hormone gene contains multiple thyroid response elements. J. Biol. Chem. 264:12063-12073.

O'Connell, M. J., C. Norbury, and P. Nurse. 1994. Premature chromatin condensation upon accumulation of NIMA. EMBO J. 13:4926-4937.

Osmani, A. H., K. O'Donnell, R. T. Pu, and S. A. Osmani. 1991a. Activation of the *nim*A protein kinase plays a unique role during mitosis that cannot be bypassed by absence of the *bim*E checkpoint. EMBO J. 10:2669-2679.

Osmani, A. H., S. L. McGuire, and S. A. Osmani. 1991b. Parallel activation of the NIMA and $p34^{cdc2}$ cell cycle-regulated protein kinases is required to initiate mitosis in A. nidulans. Cell 67:283-291.

Penman, S. 1995. Rethinking cell structure. Proc. Natl. Acad. Sci. USA 92:5251-5257.

Perlmann, T., P. N. Rangarajan, K. Umesono, and R. M. Evans. 1993. Determinants for selective RAR and TR recognition of direct repeat HREs. Genes Dev. 7:1411-1422.

Peter, M., J. Nakagawa, M. Doree, J. C. Labbe, and E. A. Nigg. 1990. In vitro disassembly of the nuclear lamina and M phase-specific phosphorylation of lamins by *cdc2* kinase. Cell 61:591-602.

Petty, K. J., B. Desvergne, T. Mitsuhashi, and V. M. Nikodem. 1990. Identification of a thyroid hormone response element in the malic enzyme gene. J. Biol. Chem. 265:7395-7400.

Pfaller, R., C. Smythe, and J. W. Newport. 1991. Assembly/disassembly of the nuclear envelope membrane: cell cycle-dependent binding of nuclear membrane vesicles to chromatin in vitro. Cell 65:209-217.

Pfaller, R., and J. W. Newport. 1995. Assembly/disassembly of the nuclear envelope membrane. J. Biol. Chem. 270:19066-19072.

Pienta, K. J., A. W. Partin, and D. S. Coffey. 1989. Cancer as a disease of DNA organization and dynamic cell structure. Cancer Res. 49:2525-2532.

Pomerance, M., F. Schweighoffer, B. Tocque, and M. Pierre. 1992. Stimulation of mitogen-activated protein kinase by oncogenic Ras-P21-GTPase-activating protein interaction. J. Biol. Chem. 267:16155-16160.

Posada, J., N. Yew, N. G. Ahn, G. F. Vande Woude, and J. A. Cooper. 1993. Mos stimulates MAP kinase in *Xenopus* oocytes and activates a MAP kinase kinase in vitro. Mol. Cell. Biol. 13:2546-2553.

Privalsky, M. L., P. Boucher, A. Koning, and C. Judelson. 1988. Genetic dissection of functional domains within the avian erythroblastosis virus *v-erb A* oncogene. Mol. Cell. Biol. 8:4510-4517.

Privalsky, M. L., M. Sharif, and K. R. Yamamoto. 1990. The viral erbA oncogene protein, a constitutive repressor in animal cells, is a hormone-regulated activator in yeast. Cell 63:1277-1286.

Privalsky, M. L. 1992. *v-erb A*, nuclear hormone receptors, and oncogenesis. Biochim. Biophys. Acta 1114:51-62.

Ptashne, M. 1988. How eukaryotic transcriptional activators work. Nature (London) 335:683-689.

Ptashne, M., and A. A. F. Gann. 1990. Activators and targets. Nature 346:329-331.

Pugh, B. F., and R. Tjian. 1992. Diverse transcriptional functions of the multisubunit eukaryotic TFIID complex. J. Biol. Chem. 267:679-682.

Ranjan, M., J. Wong, and Y.-B. Shi. 1994. Transcriptional repression of *Xenopus* TRß gene is mediated by a thyroid hormone response element located near the start site. J. Biol. Chem. 269:24699-24705.

Rascle, A., J. Ghysdael, and J. Samarut. 1994. c-ErbA, but not v-ErbA, competes with a putative erythroid repressor for binding to the carbonic anhydrase II promoter. Oncogene 9:2853-2867.

Rastinejad, F., T. Perlman, R. M. Evans, and P. B. Sigler. 1995. Structural determinants of nuclear receptor assembly on DNA direct repeats. Nature (London) 375:203-211.

Reichelt, R., A. Holzenburg, E. L. Buhle, M. Jarnik, A. Engel, and U. Aebi. 1990. Correlation between structure and mass distribution of the nuclear pore

complex and of distinct pore complex components. J. Cell Biol. 110:883-894.

Remerowski, M. L., E. Kellenbach, R. Boelens, G. A. van der Marel, J. H. van Boom, B. A. Maler, K. R. Yamamoto, and R. Kaptein. 1991. ^{1}H NMR studies of DNA recognition by the glucocorticoid receptor: complex of DNA binding domain with a half-site response element. Biochemistry 30:11620-11624.

Renkawitz, R. 1990. Transcriptional repression in eukaryotes. Trends Genet. 6:192-197.

Richardson, W. D., A. D. Mills, S. M. Dilworth, R. A. Laskey and C. Dingwall. 1988. Nuclear protein migration involves two steps: Rapid binding at the nuclear envelope followed by slower translocation through nuclear pores. Cell 52:655-664.

Ris, H., and M. Malecki. 1993. High resolution field emission scanning electron microscopy imaging of internal cell structures after Epon extraction from sections: a new approach to correlative ultrastructural and immunocytochemical studies. J. Struct. Biol. 111:148-157.

Rous, P. 1911. Transmission of a malignant new growth by means of a cell-free filtrate. J. Am. Med. Assoc. 56:198.

Rout, M. P., and S. R. Wente. 1994. Pores for thought: nuclear pore complex proteins. Trends Cell Biol. 4:357-365.

Rowe, P. M. 1995. Neural networks: a bridge between neuroscience and psychology. Mol. Med. Today 1:168-173.

Rungger, D., L. Muster, R. Boeck, and A. Nichols. 1990. Tissue-specific transactivation of the rabbit beta-globin promoter in *Xenopus* oocytes. Differentiation 44:8-17.

Saatcioglu, F., T. Deng, and M. Karin. 1993a. A novel cis element mediating ligand-independent activation by c-ErbA: Implications for hormonal regulation. Cell 75:1095-1105.

Saatcioglu, F., P. Bartunek, T. Deng, M. Zenke, and M. Karin. 1993b. A conserved C-terminal sequence that is deleted in v-ErbA is essential for the biological activities of c-ErbA (the thyroid hormone receptor). Mol. Cell. Biol. 13:3675-3685.

Sagata, N., M. Oskarsson, T. Copeland, J. Brumbaugh, and G. F. Vande Woude. 1988. Function of c-*mos* proto-oncogene product in meiotic maturation in *Xenopus* oocytes. Nature (London) 335:519-525.

Samarut, J., and L. Gazzolo. 1982. Target cells infected by avian erythroblastosis virus differentiate and become transformed. Cell 28:921-929.

Sap, J., A. Munoz, K. Damm, Y. Goldberg, J. Ghysdael, A. Leutz, H. Berg, and B. Vennström. 1986. The c-erb-A protein is a high affinity receptor for thyroid hormone. Nature (London) 324:635-640.

Sap, J., A. Munoz, J. Schmitt, H. Stunnenberg, and B. Vennström. 1989. Repression of transcription mediated by a thyroid hormone response element by the v-erb-A oncogene product. Nature (London) 340:242-244.

Sap, J., L. de Magistris, H. Stunnenberg, and B. Vennström. 1990. A major thyroid hormone response element in the third intron of the rat growth hormone gene. EMBO J. 9:887-896.

Schaufele, F., B. L. West, and T. L. Reudelhuber. 1990a. Overlapping Pit-1 and Sp1 binding sites are both essential to full rat growth hormone gene promoter activity despite mutually exclusive Pit-1 and Sp1 binding. J. Biol. Chem. 265:17189-17196.

Schaufele, F., J. A. Cassill, B. L. West, and T. L. Reudelhuber. 1990b. Resolution of diagonal gel mobility shift assays of multisubunit complexes binding to a functionally important element of the rat growth hormone gene promoter. J. Biol. Chem. 265:14592-14598.

Schaufele, F., B. L. West, and T. L. Reudelhuber. 1990c. Somatotroph- and lactotroph-specific interactions with the homeobox protein binding sites in the rat growth hormone gene promoter. Nucleic Acids Res. 18:5235-5243.

Schaufele, F., B. L. West, and J. D. Baxter. 1992. Synergistic activation of the rat growth hormone promoter by Pit-1 and the thyroid hormone receptor. Mol. Endocrinol. 6:656-665.

Scheer, U., J. Kartenbeck, M. F. Trendelenburg, J. Stadler, and W. W. Franke. 1976. Experimental disintegration of the nuclear envelope: evidence for pore-connecting fibrils. J. Cell Biol. 69:1-18.

Schliephake, A. W., and A. Rethwilm. 1994. Nuclear localization of foamy virus Gag precursor protein. J. Virology:4946-4954.

Schroeder, C., C. Raynoschek, U. Fuhrmann, K. Damm, B. Vennström, and H. Beug. 1990. The v-erbA oncogene causes repression of erythrocyte-specific genes and an immature, aberrant differentiation phenotype in normal erythroid progenitors. Oncogene 5:1445-1453.

Schroeder, C., L. Gibson, and H. Beug. 1992a. The v-*erbA* oncogene requires cooperation with tyrosine kinases to arrest erythroid differentiation induced by ligand-activated endogenous c-*erbA* and retinoic acid receptor. Oncogene 7:203-216.

Schroeder, C., L. Gibson, and H. Beug. 1992b. Modulation of normal erythroid differentiation by the endogenous thyroid hormone and retinoic acid receptors: a possible target for v-*erbA* oncogene action. Oncogene 7:217-227.

Schüle, R., K. Umesono, D. J. Mangelsdorf, J. Bolado, J. W. Pike, and R. M. Evans. 1990a. Jun-fos and receptors for vitamins A and D recognize a common response element in the human osteocalcin gene. Cell 61:497-504.

Schüle, R., P. Rangarajan, S. Kliewer, L. J. Ransone, J. Bolado, N. Yang, I. M. Verma, and R. M. Evans. 1990b. Functional antagonism between oncoprotein c-*jun* and the glucocorticoid receptor. Cell 62:1217-1226.

Schwabe, J. W. R., D. Neuhaus, and D. Rhodes. 1990. Solution structure of the DNA-binding domain of the oestrogen receptor. Nature (London) 348:458-461.

Sealy, L., G. Moscovici, C. Moscovici, and J. M. Bishop. 1983. Site-specific mutagenesis of avian erythroblastosis virus: v-erb-A is not required for transformation of fibroblasts. Virology 130:179-194.

Selmi, S., and H. H. Samuels. 1991. Thyroid hormone receptor/c-erbA and v-erbA: a single amino acid difference in the C-terminal region influences dominant negative activity and receptor dimer formation. J. Biol. Chem. 266:11589-11593.

Serfling, E., M. Jason, and W. Schaffner. 1985. Enhancers and eukaryotic gene transcription. Trends Genet. 1:224-230.

Sharif, M., and M. L. Privalsky. 1991. *v-erb A* oncogene function in neoplasia correlates with its ability to repress retinoic acid receptor action. Cell 66:885-893.

Sheets, M. D., M. Wu, and M. Wickens. 1995. Polyadenylation of c-*mos* mRNA as a control point in *Xenopus* meiotic maturation. Nature (London) 374:511-516.

Shibuya, E. K., A. J. Polverino, E. Chang, M. Wigler, and J. V. Ruderman. 1992. Oncogenic Ras triggers the activation of 42-kDa mitogen-activated protein kinase in extracts of quiescent *Xenopus* oocytes. Proc. Natl. Acad. Sci. USA 89:9831-9835.

Siomi, H., and G. Dreyfuss. 1995. A nuclear localization domain in the hnRNP A1 protein. J. Cell Biol. 129:551-560.

Sippel, A. E., and R. Renkawitz. 1989. The chicken lysozyme gene. In R. Renkawitz (ed.) *Tissue Specific Gene Expression*. New York, VCH Publishers. p. 185-198.

Sjöberg, M., and B. Vennström. 1995. Ligand-dependent and independent transactivation by thyroid hormone receptor ß2 is determined by the structure of the hormone response element. Mol. Cell. Biol. 15:4718-4726.

Slater, E. P., O. Rabenau, M. Harin, J. D. Baxter, and M. Beato. 1985. Glucocorticoid receptor binding and activation of a heterologous promoter by dexamethasone by the first intron of the human growth hormone gene. Mol. Cell. Biol. 5:2984-2992.

Smith, L. D. 1989. The induction of oocyte maturation: Transmembrane signaling events and regulation of the cell cycle. Development 107:685-699.

Smit-McBride, Z., and M. L. Privalsky. 1993. Functional domains of the *v-erbA* protein necessary for oncogenesis are required for transcriptional activation in *Saccharomyces cerevisiae*. Oncogene 8:1465-1475.

Spivack, J. G., R. L. Erikson, and J. L. Maller. 1984. Microinjection of $pp60^{v\text{-}src}$ into *Xenopus* oocytes increases phosphorylation of ribosomal protein S6 and accelerates the rate of progesterone-induced meiotic maturation. Mol. Cell. Biol. 4:1631-1634.

Stafstrom, J. P., and A. Staehelin. 1984. Are annulate lamellae in the *Drosophila* embryo the result of overproduction of nuclear pore components? J. Cell Biol.

98:699-708.

Steinert, G., E. Baltus, J. Hanocq-Quertier, and J. Brachet. 1974. Ultrastructure of Xenopus laevis oocytes after injection of an extract from progesterone-treated oocytes. J. Ultrastruct. Res. 49:188-210.

Stick, R., and P. Hausen. 1985. Changes in the nuclear lamina composition during early development of Xenopus laevis. Cell 41:191-200.

Stochaj, U., and P. Silver. 1992. Nucleocytoplasmic traffic of proteins. Eur. J. Cell Biol. 59:1-11.

Subauste, J. S., and R. J. Koenig. 1995. Comparison of the DNA binding specificity and function of v-ErbA and thyroid hormone receptor α1. J. Biol. Chem. 270:7957-7962.

Teich, N., J. Wyke, and P. Kaplan. 1985. Pathogenesis of retrovirus-induced disease. In R. Weiss, N. Teich, H. Varmus, and J. Coffin (eds.) *RNA Tumor Viruses*. 2nd edition. Supplements and appendixes. New York, Cold Spring Harbor Press. p. 187-248.

Temin, H. M. 1988. Evolution of cancer genes as a mutation-driven process. Cancer Res. 48:1697-1701.

Temin, H. M. 1989. Retrovirus variation and evolution. Genome 31:17-22.

Theulaz, I., R. Hipskind, B. ten Heggeler-Bordier, S. Green, V. Kumar, P. Chambon, and W. Wahli. 1988. Expression of human estrogen receptor mutants in *Xenopus* oocytes: correlation between transcriptional activity and ability to form protein-DNA complexes. EMBO J. 7:1653-1660.

Thompson, C. C., and R. M. Evans. 1989. Trans-activation by thyroid hormone receptors: Functional parallels with steroid hormone receptors. Proc. Natl. Acad. Sci. USA 86:3494-3498.

Tjian, R., and T. Maniatis. 1994. Transcriptional activation: a complex puzzle with few easy pieces. Cell 77:5-8.

Toney, J. H., L. Wu, A. E. Summerfield, G. Sanyal, B. M. Forman, J. Zhu, and H. H. Samuels. 1993. Conformational changes in chicken thyroid hormone receptor α1 induced by binding to ligand or to DNA. Biochemistry 32:2-6.

Török, I., D. Strand, R. Schmitt, G. Tick, T. Török, I. Kiss, and B. M. Mechler. 1995. The *overgrown hematopoietic organs-31* tumor suppressor gene of

Drosophila encodes an *importin*-like protein accumulating in the nucleus at the onset of mitosis. J. Cell Biol. 129:1473-1489.

Umesono, K., and R. M. Evans. 1989. Determinants of target gene specificity for steroid/thyroid hormone receptors. Cell 57:1139-1146.

Umesono, K., K. K. Murakami, C. C. Thompson, and R. M. Evans. 1991. Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D3 receptors. Cell 65:1255-1266.

Varmus, H. 1989. An historical overview of oncogenes. In Weinberg, R. A. (ed.) *Oncogenes and the Molecular Origins of Cancer.* Monograph 18. New York, Cold Spring Harbor Press. p.3-44.

Vennström, B., and J. M. Bishop. 1982. Isolation and characterization of chicken DNA homologous to the two putative oncogenes of avian erythroblastosis virus. Cell 28:135-143.

Verneuil, H., and D. Metzger. 1990. The lack of transcriptional activation of the v-erbA oncogene is in part due to a mutation present in the DNA binding domain of the protein. Nucleic Acids Res. 18:4489-4497.

Waldmann, R., P. Hanson, and H. Schulman. 1990. Multifunctional Ca^{2+}/calmodulin-dependent protein kinase made Ca^{2+}-independent for functional studies. Biochemistry 29:1679-1684.

Wang, H.-C., B. Beer, D. Sassano, A. J. Blume, and M. R. Ziai. 1991. Gene expression in *Xenopus* oocytes. Int. J. Biochem. 23:271-276.

Wasserman, W. J., and Y. Masui. 1975. Effects of cyclohexamide on a cytoplasmic factor initiating meiotic maturation in *Xenopus* oocytes. Exp. Cell Res. 91:381-388.

Watson, C. S. 1991. Human estrogen receptor introduced into the Xenopus oocyte represses expression from an artificial frog estrogen response element. J. Steroid Biochem. Molec. Biol. 38:423-431.

Weinberg, R. A. (ed.) 1989a. *Oncogenes and the Molecular Origins of Cancer.* Monograph 18. New York, Cold Spring Harbor Laboratory Press. 365 pp.

Weinberg, R. A. 1989b. Oncogenes and multistep carcinogenesis. In Weinberg, R. A. (ed.) *Oncogenes and the Molecular Origins of Cancer.* Monograph 18. New York, Cold Spring Harbor Press. p. 307-326.

Weinberger, C., C. C. Thompson, E. S. Ong, R. Lebo, D. J. Grvol, and R. M. Evans. 1986. The c-erbA gene encodes a thyroid hormone receptor. Nature (London) 324:641-646.

West, B. L., D. F. Catanzaro, S. A. Mellon, P. A. Cattini, J. D. Baxter, and T. L. Reudelhuber. 1987. Interaction of a tissue-specific factor with an essential rat growth hormone promoter element. Mol. Cell. Biol. 7:1193-1197.

Wills, J. W., and R. C. Craven. 1991. Form, function, and use of retroviral Gag proteins. AIDS:639-654.

Wong, J., and Y.-B. Shi. 1995. Coordinated regulation of and transcriptional activation by *Xenopus* thyroid hormone and retinoid X receptors. J. Biol. Chem. 270:18479-18483.

Wong, J., Y.-B. Shi, and A. P. Wolffe. 1995. A role for nucleosome assembly in both silencing and activation of the *Xenopus* TRßA gene by the thyroid hormone receptor. Genes Dev. 9:2696-2711.

Xu, L., W. Schaffner, and D. Rungger. 1993. Transcriptional activation by recombinant GAL4-VP16 in the *Xenopus* oocyte. Nucleic Acids Res. 21:2775.

Yamamoto, T., H. Hihara, T. Nishida, S. Kawai, T. Ooi, and K. Toyoshima. 1983. A new avian erythroblastosis virus, AEV-H, carries *erbB* gene responsible for the induction of both erythroblastosis and sarcomas. Cell 34:225-232.

Ye, X. S., G. Xu, R. T. Pu, R. R. Fincher, S. L. McGuire, A. H. Osmani, and S. A. Osmani. 1995. The NIMA protein kinase is hyperphosphorylated and activated downstream of $p34^{cdc2}$/cyclin B: coordination of two mitosis promoting kinases. EMBO J. 14:986-994.

Ye, Z.-S., B. M. Forman, A. Aranda, A. Pascual, H.-Y. Park, J. Casanova, and H. H. Samuels. 1988. Rat growth hormone gene expression: both cell-specific and thyroid hormone response elements are required for thyroid hormone regulation. J. Biol. Chem. 263:7821-7829.

Yen, P. M., M. Ikeda, J. H. Brubaker, M. Forgione, A. Sugawara, and W. W. Chin. 1994. Roles of v-erbA homodimers and heterodimers in mediating dominant negative activity by v-erbA. J. Biol. Chem. 269:903-909.

Yen, P. M., and W. W. Chin. 1994. Molecular mechanisms of dominant negative activity by nuclear hormone receptors. Mol. Endocrinol. 8:1450-1454.

Yew, N., M. L. Mellini, and G. F. Vande Woude. 1992. Meiotic initiation by the *mos* protein in *Xenopus*. Nature (London) 355:649-652.

Yu, V. C., C. Delsert, B. Andersen, J. M. Holloway, O. V. Devary, A. M. Näär, S. Y. Kim, J.-M. Boutin, C. Glass, and M. G. Rosenfeld. 1991. RXRß: a coregulator that enhances binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements. Cell 67:1251-1266.

Zechel, C., X.-Q. Shen, P. Chambon, and H. Gronemeyer. 1994a. Dimerization interfaces formed between the DNA binding domains determine the cooperative binding of RXR/RAR and RXR/TR heterodimers to DR5 and DR4 elements. EMBO J. 13:1414-1424.

Zechel, C., X.-Q. Shen, J.-Y. Chen, Z.-P. Chen, P. Chambon, and H. Gronemeyer. 1994b. The dimerization interfaces formed between the DNA binding domains of RXR. RAR and TR determine the binding specificity and polarity of the full-length receptors to direct repeats. EMBO J. 13:1425-1433.

Zenke, M., P. Kahn, C. Disela, B. Vennström, A. Leutz, K. Keegan, M. J. Hayman, H. R. Choi, N. Yew, J. D. Engel, and H. Beug. 1988. V-erbA specifically suppresses transcription of the avian erythrocyte anion transporter (band 3) gene. Cell 52:107-119.

Zenke, M., A. Munoz, J. Sap, B. Vennström, and H. Beug. 1990. v-erbA oncogene activation entails the loss of hormone-dependent regulator activity of c-erbA. Cell 61:1035-1049.

Zhang, X.-K., K. N. Wills, M. Husmann, T. Hermann and M. Pfahl. 1991. Novel pathway for thyroid hormone receptor action through interaction with *jun* and *fos* oncogene activities. Mol. Cell. Biol. 11:6016-6025.

Zhang, X.-K., B. Hoffman, P. B.-V. Tran, G. Graupner, and M. Pfahl. 1992. Retinoid X receptor is an auxiliary protein for thyroid hormone and retinoic acid receptors. Nature (London) 355:441-446.

Appendix 1

The results of the investigation described in Chapter II were published and a reprint of the publication is included in Appendix 1.

In consultation with my supervisor, Dr. Lizabeth A. Allison, I designed and carried out the study described in this publication. Colleen C. Nelson prepared the TR C122>A clone and some of the reporter gene constructs while she was a postdoctoral fellow in the laboratory of Paul J. Romaniuk.

Sylvia B. Nagl

Constitutive Transactivation by the Thyroid Hormone Receptor and a Novel Pattern of Activity of its Oncogenic Homolog v-*Erb*A in *Xenopus* Oocytes

Sylvia B. Nagl*, Colleen C. Nelson†, Paul J. Romaniuk, and Lizabeth A. Allison

Department of Zoology (S.B.N., L.A.A.)
University of Canterbury
Christchurch, New Zealand

Department of Biochemistry and Microbiology (C.C.N., P.J.R.)
University of Victoria
Victoria, British Columbia, Canada V8W 3P6

In *Xenopus* oocytes, the rat thyroid hormone receptor α (rTRα), but not its oncogenic homolog v-*Erb*A, constitutively activated thyroid hormone (T_3)-responsive reporter genes at four positive thyroid hormone-responsive elements (TREs). At a subset of the positive TREs tested, the addition of T_3 resulted in a further enhancement of reporter gene activation. In contrast, both rTRα and v-*Erb*A functioned as constitutive activators when bound to the clone 122 TREs, which are induced by unliganded TR in mammalian cells. Therefore, the responses of the ligand-independent activation domains of TR and v-*Erb*A to cell-specific and TRE-mediated induction are not equivalent. Coexpression of the human retinoid X receptor α (hRXRα) enhanced both ligand-dependent and ligand-independent activation functions of rTRα and human TRβ (hTRβ) at a palindromic TRE (TREp). An endogenous TR activity mediated T_3 induction of TREp, while being repressed by an *in vitro*-generated dominant negative mutant of TR. T_3-mediated gene activation, by exogenous or endogenous TR, was repressed by v-*Erb*A at three positive TREs, but not at the TRE from the third intron of the rat GH gene (rGH$_3$TRE). Interestingly, preinjection of nuclear protein extract from anterior pituitary cells converted v-*Erb*A into a constitutive activator at rGH$_3$TRE. The pituitary-specific factor Pit-1/GHF-1 or hRXRα did not induce activation by v-*Erb*A at rGH$_3$TRE, suggesting that the dominant negative phenotype of v-*Erb*A can be abolished by direct or indirect interactions with other nuclear factors. (Molecular Endocrinology 9: 1522–1532, 1995)

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INTRODUCTION

Thyroid hormone receptors (TRs) belong to the superfamily of nuclear hormone receptors, which includes receptors for steroids, retinoids, and vitamin D_3, and act as ligand-inducible transcription factors (1). TRs are encoded by two genes, c-*erb*Aα and c-*erb*Aβ, which are cellular homologs of the retroviral v-*erb*A oncogene involved in avian erythroblastosis (Refs. 2 and 3; for reviews, see Refs. 4 and 5). TRs, like other transcription factors, are composed of modular functional domains whose action is dependent on cellular context. TRs control expression of T_3-responsive genes at *cis*-acting DNA control sequences termed thyroid hormone-responsive elements (TREs) which possess diverse sequence characteristics (for a review, see Ref. 4). Induction of gene transcription is achieved through autonomous activation domains: a constitutive activation domain located in the N-terminal region of the receptor and a ligand-dependent activation domain located in the C-terminal part (for a review and references, see Ref. 6). Although transcriptional activation by TR is primarily ligand-dependent in mammalian cells, ligand-independent activation has also been observed (7–10). In the yeast, *Saccharomyces cerevisiae*, TR functions as a ligand-independent activator with enhanced expression in the presence of T_3 (11). In mammalian cells, unliganded TRs, thought to be constitutively bound to hormone response elements, can suppress the basal activity of promoters containing TREs (12–17). The repressor function has been mapped to the hinge domain of TR and was shown to be separable from its activator function (18). A C-terminal silencer domain has also been characterized (12, 19); however, how the activation and repression domains are regulated in the context of the full-length receptor is presently not well understood.

At positively regulated TREs, addition of thyroid hormone (T_3) results in the activation of transcription, suggesting that the hormone triggers a conformational change in TR that induces the activator function (for a review, see Ref. 5). At negatively regulated TREs, TRs act as transcriptional repressors when bound to T_3 (for reviews, see Refs. 4 and 5). In addition, two novel TREs that mediate strong activation by unliganded TR were recently identified, whereas addition of T_3 reverses this response (9, 10). Although TRs are able to bind TREs as monomers and homodimers, heterodimerization with auxiliary nuclear factors, notably retinoid X receptors (RXRs), results in increased affinity for the response element *in vitro* and enhanced activation of gene expression *in vivo* (Ref. 20; for a review, see Ref. 21).

The retroviral v-*erb*A oncogene, a highly mutated version of chicken TRα, acts as a dominant repressor in mammalian and avian cells, blocking activation of gene expression by liganded TRs (Refs. 14 and 16; for a review, see Ref. 22). Mutations within the C terminus of the v-*erb*A protein appear primarily responsible for its conversion into a constitutive transcriptional repressor, as these mutations result in loss of the ability to bind thyroid hormone at high affinity (Refs. 14, 16, and 17; for a review, see Ref. 22). v-*Erb*A has retained some binding ability, since, surprisingly, v-*Erb*A acts as a hormone-regulated activator in *S. cerevisiae* (11, 23). Two mutations within the DNA- binding domain, and mutations in an N-terminal region outside of the DNA binding domain, alter the range of DNA target sequences the v-*erb*A protein can bind to relative to TR (Refs. 17, and 24–26; for a review, see Ref. 22).

Oocytes of the frog *Xenopus laevis* are an exceptionally efficient assay system to characterize the function of both *cis*- and *trans*-acting factors in gene expression (27). Unlike expression studies in transfected cultured cells, oocyte microinjection offers the possibility to directly introduce cell components, such as nuclear proteins, as well as gene templates. Here, we show that oocytes constitute a viable environment for the reconstitution of complex regulatory networks, involving nuclear hormone receptors. TR, expressed from cloned genes microinjected into oocytes, functioned as a ligand-independent activator of reporter genes under the control of five different TREs. At a subset of the positive TREs tested, the addition of T_3 led to a further increase in reporter gene expression. Both the α- and β-isoform of TR acted as constitutive activators at a palindromic TRE in oocytes, while coexpression of RXRα enhanced both ligand-independent and ligand-dependent activation. Moreover, T_3 did not inhibit the ligand-independent activation function of TR at a recently identified TRE, which is strongly induced by TR without T_3 and repressed with T_3 in mammalian cells (clone 122 TREs). In addition, the v-*erb*A protein acted as a constitutive activator at the clone 122 TREs. In the absence of exogenous TR, a reporter gene regulated by a palindromic TRE was weakly induced by endogenous TR. Induction by endogenous TR was dominantly repressed by v-*Erb*A or a dominant negative *in vitro*-generated mutant of human TRβ (hTRβ). At three positive TREs, v-*Erb*A acted as a dominant repressor of TR action; however, v-*Erb*A did not repress basal gene transcription of T_3-responsive genes. The v-*erb*A protein dominantly repressed TR function at a direct repeat, a palindromic and an inverted palindromic TRE, but did not affect TR regulation of the tripartite TRE from the third intron of the rat GH gene (rGH_3TRE). After preinjection of nuclear protein extract from anterior pituitary cells, v-*Erb*A functioned as a constitutive activator at rGH_3TRE. RXR or the pituitary-specific transcription factor, Pit-1/GHF-1, when expressed from coinjected templates, did not convert the v-*erb*A protein to an activator at this TRE. This suggests that other factors, which can induce the constitutive activator function of v-*Erb*A at rGH_3TRE, are present in nuclear protein extracts from anterior pituitary cells. These findings exemplify the utility of *Xenopus* oocytes for the characterization of cell-specific actions of nuclear receptor domains.

RESULTS

Both Constitutive and T_3-Mediated Gene Activation Functions of TRα and TRβ Are Induced in *Xenopus* Oocytes and Enhanced by RXR

We sought to establish whether *Xenopus* oocytes can be used for the reconstitution of nuclear receptor networks *in vivo*. In this context, we wished to ascertain whether oocytes provide a cellular background for nuclear hormone receptor action similar to either yeast or mammalian cells or whether they constitute a unique context. TR mRNA is present at all stages of oogenesis (28) and in fertilized eggs (29); however, at the onset of our study, TR protein had not been detected by immunoprecipitation or Western immunoblotting (29), suggesting that maternal transcripts are stored for use later in embryogenesis. Results obtained by enzyme-linked immunosorbent assay (ELISA) showed that oocytes contain no detectable endogenous T_3 (<0.1 nM). After incubation in 100 nM T_3, oocytes exhibited an intracellular hormone concentration of ~5 nM (data not shown), which falls within the physiological range for T_3 action. Thus, we presumed that *Xenopus* oocytes would be suitable for experiments investigating transcriptional regulation by exogenous TR and v-*erb*A protein at a range of diverse TREs, both with and without added T_3.

TREs consist of six-base pair consensus sequences, termed half-sites. These half-sites can be oriented as imperfect inverted, direct, or divergent repeats, separated by nucleotide gaps of variable size (for a review, see Ref. 4). Naturally occurring promoters often show mixtures of half-sites in differing orientations. We sought to reflect the natural diversity of TREs by employing four positively regulated response

elements possessing different half-site characteristics; *i.e.* the direct repeat TRE in the rat malic enzyme gene promoter (malTRE) (30), the inverted palindromic TRE in the promoter region of the chicken lysozyme gene (lysTRE) (13), rGH_3TRE (31), a synthetic palindromic TRE (TREp) (14), and one complex response element which is strongly induced by unliganded TR and repressed by T_3 in mammalian cells (clone 122 TREs) (10).

A rat TRα (rTRα) expression plasmid and a chloramphenicol acetyltransferase (CAT) reporter gene under the control of one of five different TREs were microinjected into the oocyte nucleus. Unliganded rTRα induced transcription of all five TRE-regulated CAT reporter genes (Fig. 1). Constitutive reporter gene induction by rTRα was strongest when under the control of lysTRE, TREp, or the clone 122 TREs. CAT reporter gene transcription under control of three positively regulated TREs (malTRE, lysTRE, TREp) was further enhanced by the addition of T_3 but was unresponsive to hormone with rGH_3TRE (Fig. 1). In contrast to results obtained in CV1 (monkey kidney) cells (10), addition of T_3 did not significantly repress TR-mediated transcription regulated by the clone 122 TREs (Fig. 1). Reporter gene induction by TR was shown to

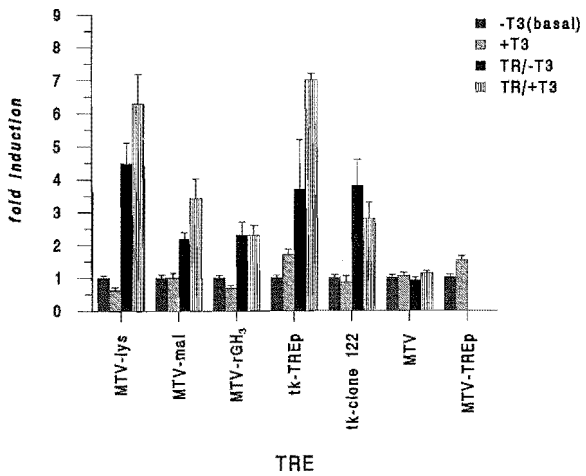

Fig. 1. TR-Mediated TRE-CAT Reporter Gene Transcription in *Xenopus* Oocytes

Two nanograms of ΔMTV-CAT or *tk*-CAT reporter gene construct linked to one of five different TREs as indicated or 2 ng ΔMTV-CAT lacking a TRE were microinjected into the oocyte nucleus together with 0.5 ng of the rTRα expression vector. Oocytes were cultured in the presence or absence of 100 nM T_3 (TR/+T_3 and TR/−T_3) for 24 h. Alternatively, oocytes were microinjected with reporter constructs alone and incubated with and without hormone (+T_3/−T_3). ΔMTV-TREp-CAT was tested with and without T_3 only. Activation of the reporter gene was measured in terms of the levels of CAT protein present in oocytes, analyzed individually by ELISA. Basal transcription levels of the reporter constructs in the absence of exogenous TR and T_3 were arbitrarily assigned the value 1. For each CAT reporter construct two or three independent experiments (five oocytes per treatment) were performed, and the mean fold induction over basal transcription was calculated. The error bars indicate the SEMS.

be dependent on the presence of a TRE in oocytes, as expression levels of the mouse mammary tumor virus (ΔMTV)-CAT reporter gene lacking a TRE sequence were not induced by coinjection of rTRα, in the presence or absence of T_3 (Fig. 1).

Unexpectedly, there was a weak T_3-dependent induction of the thymidine kinase (*tk*)-TREp-CAT and TREp-ΔMTV-CAT reporter constructs in the absence of exogenous TR, while the ΔMTV promoter alone was not responsive to T_3 (Fig. 1). Contrary to our original assumptions, these results suggested the presence of low levels of an endogenous TR-like activity in oocytes. A recent study has now reported that *Xenopus* oocytes contain both TRα and TRβ protein (32). The observed T_3-mediated induction of a CAT reporter gene under control of TREp in the absence of exogenous TR indicates that these endogenous TRs can function as T_3-regulated activators.

The β-isoform of the receptor, hTRβ, also constitutively induced transcription at TREp, albeit to a lesser degree than rTRα (Fig. 2). Gene activation by hTRβ was not significantly enhanced in the presence of hormone at TREp. Moreover, to achieve detectable levels of reporter gene induction by hTRβ, a 5 times greater amount of the expression vector (RSh-TRβ) had to be microinjected compared with RS-rTRα. This increased requirement might have been due to differences in the expression of the two constructs in oocytes or to the decreased binding affinity of TRβ for palindromic TREs (11, 33).

RXRs have recently been shown to heterodimerize with TRs, enhancing TR binding to TRE elements and augmenting T_3-mediated transcriptional activation (for

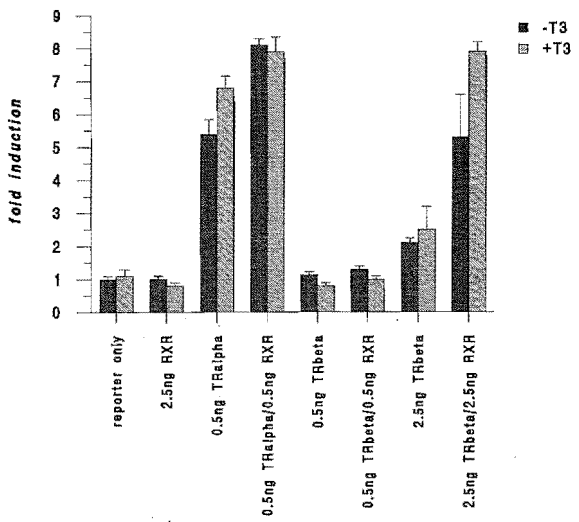

Fig. 2. *tk*-TREp-CAT Reporter Gene Transcription in the Presence of hRXRα and rTRα or hTRβ

Oocytes were microinjected with 2 ng *tk*-TREp-CAT only (reporter only), or together with varying amounts of hRXRα (RXR), rTRα (TRalpha) and hTRβ (TRbeta) expression plasmids as indicated. Oocytes were incubated with and without hormone (+T_3, −T_3) for 24 h and then analyzed individually by ELISA as described in Fig. 1.

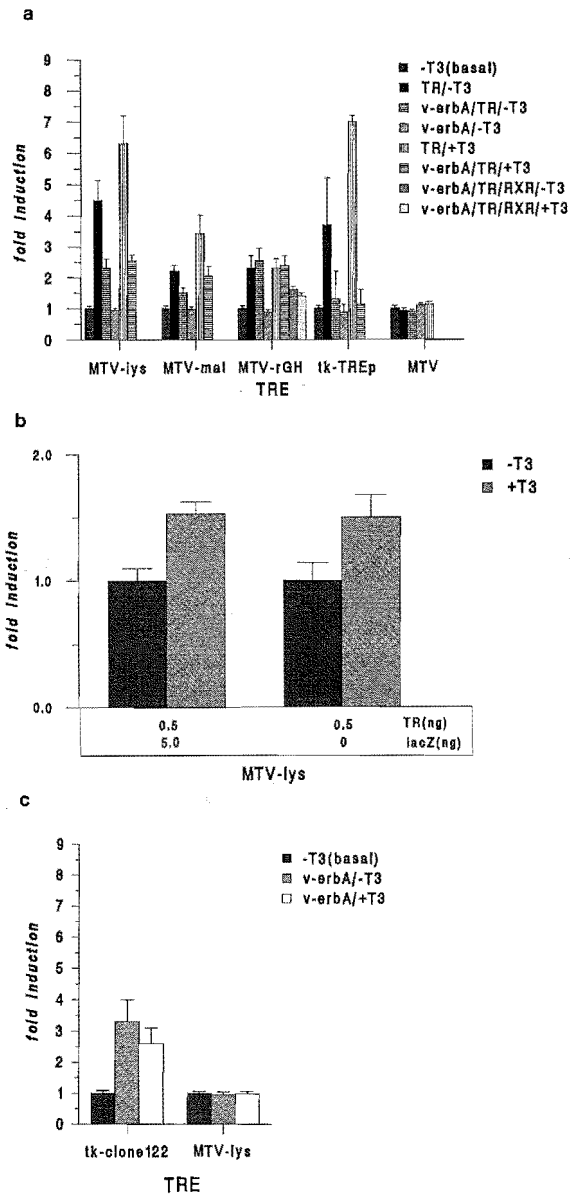

Fig. 3. Effects of v-*Erb*A on TRE-CAT Reporter Gene Transcription

A, Dominant repression of TR-mediated TRE-CAT reporter gene transcription by v-*Erb*A in *Xenopus* oocytes. Oocytes were cultured in the presence or absence of 100 nM T_3 after microinjection with 2 ng ΔMTV-CAT or *tk*-CAT reporter gene construct linked to four different TREs as indicated, or 2 ng ΔMTV-CAT without a TRE, and 0.5 ng rTRα expression vector alone (TR/+T_3, TR/−T_3), or together with a 5-fold excess of v-*erb*A (2.5 ng) (v-erbA/TR/+T_3, v-erbA/TR/−T_3). Two nanograms of rGH_3TRE reporter construct plus 0.5 ng rTRα, 2.5 ng v-*erb*A, and 2.5 ng hRXRα expression plasmids were also tested (v-erbA/TR/RXR/−T_3, v-erbA/TR/RXR/+T_3). In addition, 2 ng TRE-CAT reporter gene construct were microinjected into the oocyte nucleus with 2.5 ng v-*erb*A expression plasmid, and oocytes were cultured without T_3 (v-erbA/−T_3). Alternatively, oocytes were microinjected with reporter constructs alone and incubated in the absence of T_3 (−T3). After 24 h, oocytes were assayed individually for CAT protein by ELISA. Basal transcription rates of the CAT reporter constructs in the absence of exogenous TR, v-*Erb*A, and T_3 (−T_3) were arbitrarily assigned the value 1. Two or three independent experiments (five oocytes per treatment) were performed for each TRE-CAT reporter gene construct, and the mean fold induction over basal transcription was calculated. The error bars indicate the SEMs. B, T_3 induction of lysTRE-CAT reporter gene transcription in the presence of rTRα and *lacZ* expression plasmids. Oocytes were microinjected with 2 ng ΔMTV-lysTRE-CAT reporter gene construct plus 0.5 ng rTRα with or without 5 ng *lacZ* expression vector, as indicated, and incubated in the presence or absence of 100 nM T_3 (−T_3/+T_3). A single experiment with five oocytes per treatment was performed and analyzed as described for panel A. C, Constitutive *trans*-activation by v-*Erb*A at the clone 122 TREs. Oocytes were cultured with and without T_3 after microinjection of 2 ng pTPT-CAT plasmid, containing the clone 122 TREs linked to a *tk*-CAT reporter construct (tk-clone 122), or 2 ng ΔMTV-lysTRE-CAT plasmid, and 0.5 ng v-*erb*A expression vector (v-*erb*A/+T_3, v-*erb*A/-T_3). Alternatively, oocytes were microinjected with reporter constructs alone and incubated in the absence of T_3 (−T_3). Three independent experiments with five oocytes per treatment were performed and analyzed as described for panel A.

a review, see Ref. 21). Therefore, it was of interest to test how heterodimerization with RXR affects the action of TR in oocytes. In this series of experiments, equimolar amounts of expression plasmids for human RXRα (hRXRα) and rTRα, or hTRβ, were microinjected into the oocyte nucleus, together with the TREp-*tk*-CAT reporter construct. As shown in Fig. 2, hRXRα enhanced constitutive reporter gene activation by both rTRα and hTRβ. Coexpression of hRXRα increased T_3-independent and T_3-mediated reporter gene activation by rTRα to the same level. The presence of hRXRα enhanced the constitutive activator function of hTRβ (2.5 ng expression vector microinjected) to levels seen with unliganded rTRα alone (0.5 ng expression vector). In addition, with hRXRα present, hTRβ activated the reporter gene in a T_3-responsive manner to the same level as rTRα.

To summarize, both rTRα and hTRβ constitutively activated reporter gene transcription at five different TREs, while activation was further inducible by T_3 at a subset of TREs. Furthermore, coexpression of hRXRα enhanced both ligand-independent and ligand-dependent activation by rTRα and hTRβ at TREp. An endogenous TR activity mediated weak T_3 induction of TREp.

Only Certain Naturally Occurring TREs Act as Control Elements for Repression by v-*erb*A in the presence or absence of T_3

In mammalian and avian cells, TR activities are blocked by v-*Erb*A acting as a dominant repressor at the level of the TRE. However, this function of v-*Erb*A is not universal but depends on cellular context (11, 26). Potent suppression of basal transcription of T_3-regulated genes in the absence of TR and T_3 has also been shown in mammalian cells (14, 18) and has been implicated in v-*Erb*A oncogenesis (17, 22, 23, 34). We

sought to establish the action of v-*Erb*A in *Xenopus* oocytes in two ways. The first series of experiments tested the competitive interaction between v-*Erb*A and rTRα at naturally occurring TREs, by determining the extent to which v-*Erb*A was able to dominantly repress TR-induced activation of the reporter gene constructs. The second set of experiments tested the ability of v-*Erb*A to suppress basal promoter activity of the reporter genes in the absence of TR and T_3.

We carried out a series of coinjection experiments to test the dominant repressor function of v-*Erb*A. In both the presence and absence of T_3, v-*Erb*A acted as a dominant repressor of rTRα-mediated CAT reporter gene transcription from lysTRE, malTRE, and TREp (Fig. 3A). Repression was most complete at TREp. The dominant action of v-*Erb*A was dose-dependent, as a 5-fold excess of the v-*erb*A expression plasmid (Fig. 3A) produced an even greater repression of TR-mediated induction of the reporter genes than an equal ratio of v-*erb*A and TR templates (data not shown). In contrast, v-*Erb*A did not repress rTRα-mediated transcription under the control of rGH$_3$TRE (Fig. 3A). However, coexpression of the cofactor hRXRα reduced transcription in the presence of rTRα and v-*Erb*A at this TRE (Fig. 3A).

In mammalian cells, v-*Erb*A represses basal transcription of T_3-responsive genes in the absence of T_3 (14, 18). In the present study, v-*Erb*A did not act as a repressor of basal gene transcription under control of four different positively regulated TREs in oocytes (Fig. 3A). Repression of basal transcription by v-*Erb*A would have been detectable by the CAT ELISA employed, since the linear range of the assay (tested down to A_{405} ~0.15) extended below the CAT protein levels observed due to basal promoter activity (A_{405} ~0.23–0.3) (data not shown). The loss of this function by v-*Erb*A parallels the lack of repression of basal transcription by unliganded exogenous TR in oocytes. Importantly, expression of v-*erb*A in oocytes abolishes its function as a repressor of basal promoter activity but does not interfere with its dominant repressor potential.

We showed that dominant repression by v-*Erb*A in the oocyte is a specific effect in the following ways. First, transcription levels of the ΔMTV-CAT reporter plasmid were unaffected by the presence of rTRα and-or v-*Erb*A with and without T_3, showing that gene regulation by rTRα and v-*Erb*A is dependent on the presence of a TRE (Fig. 3A). Second, T_3-dependent induction by rTRα of the ΔMTV-lysTRE-CAT reporter gene was unaffected by the coinjection of the *lacZ* expression vector in place of the v-*erb*A construct (Fig. 3B). Both in the presence and absence of the *lacZ* vector, T_3 enhanced reporter gene expression 1.5-fold over levels induced by unliganded rTRα (Fig. 3B). This control was performed at a 2-fold greater concentration of the *lacZ* vector (5 ng) compared with experiments using v-*erb*A construct (2.5 ng). Dominant repression by v-*Erb*A was thereby shown not to be caused by 'nonspecific squelching' (35); *i.e.* essential transcription factors are not simply being titrated by the presence of an excess of v-*erb*A expression template.

The v-*erb*A protein binds T_3 only at very low affinity, due to several mutations in the C-terminal domain (14, 16, 36, 37). Presumably, this lack of hormone binding would render v-*Erb*A highly resistant to undergo a ligand-induced conformational change. For this reason, we were interested to ascertain how v-*Erb*A acts at the clone 122 TREs in oocytes. In mammalian cells, T_3 inhibits the activator function and induces the repressor function of TR at this TRE (10). In Fig. 1 we showed that, in oocytes, TR constitutively activated transcription at the clone 122 TREs. We also observed constitutive *trans*-activation of the reporter gene construct by v-*Erb*A (Fig. 3C). This induction by v-*Erb*A was not significantly affected by T_3. The v-*erb*A protein was also unresponsive to T_3 at lysTRE in oocytes (Fig. 3C).

In the previous section we reported that there was a weak T_3-dependent induction of the TREp-CAT reporter gene in the absence of exogenous TR, suggesting the presence of low levels of T_3-regulated endogenous TR in oocytes. The T_3-dependent reporter gene induction by endogenous TR was dominantly repressed to the same level by v-*Erb*A and a dominant negative *in vitro*-generated mutant of hTRβ (TR C122 > A) (Fig. 4). This DNA-binding deficient mutant is thought to repress TR-mediated gene transcription by forming inactive dimers with TR (24). Therefore, dominant repression by TR C122 > A suggests that endogenous TR was able to dimerize with the exogenous mutant TRβ. The slight T_3 dependence of transcription in the presence of TR C122 > A was interpreted as not biologically significant because of high sample variability. Repression of the reporter gene by v-*Erb*A or TR C122 > A was dependent on the presence of a TRE. The *lacZ* reporter gene, which lacks a TRE sequence, was expressed at levels similar to those of the CAT reporter gene and was not repressed by either v-*Erb*A or TR C122 > A (Fig. 4). In summary, our results show that v-*Erb*A displays an altered pattern of activity in oocytes, compared with both mammalian cells and the yeast, *S. cerevisiae*. v-*Erb*A did not repress basal reporter gene transcription yet acted as a dominant repressor of TR action at the positively regulated TREs tested, with the exception of rGH$_3$TRE. At the clone 122 TREs, v-*Erb*A acted as a constitutive activator.

Pituitary-Specific Transcriptional Regulation of rGH$_3$TRE by rTRα and v-*ErbA*

In Fig. 1 we showed that the T_3-dependent activator function of rTRα was not induced at rGH$_3$TRE in the presence of hormone. Previous studies have shown that optimal transcription of the rat GH gene (rGH) under control of TRE sequences in the promoter region is dependent on a cell-specific factor, Pit-1/GHF-1, binding at upstream elements (38). Since the

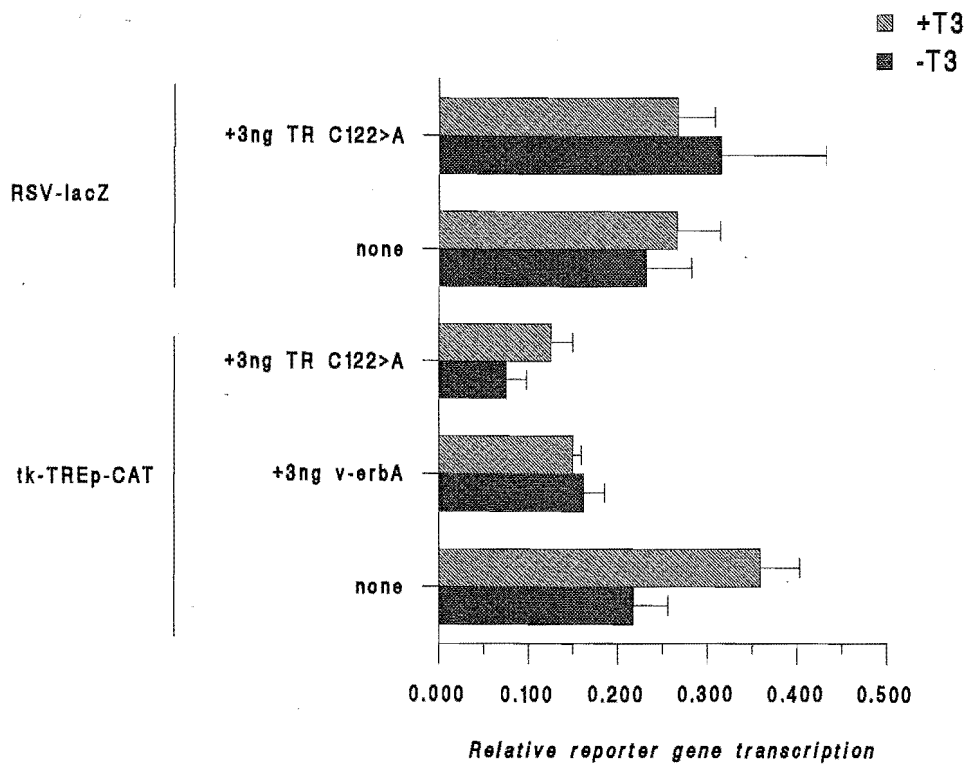

Fig. 4. Dominant Repression of an Endogenous TR-Like Activity by v-*Erb*A or an *in Vitro*-Generated Mutant of TR

Oocytes were microinjected with 2 ng *tk*-TREp-CAT reporter plasmid alone (none), or together with v-*erb*A or TR C122 > A expression plasmids in the presence or absence of 100 nM T_3 ($+T_3$, $-T_3$) in the combinations indicated. As a control, 2 ng RSV-*lacZ* reporter gene instead of *tk*-TREp-CAT construct were coinjected with TR C122 > A or injected alone (none). Relative reporter gene transcription was expressed in arbitrary units based on measurements of CAT or β-galactosidase protein levels by ELISA. Two independent experiments with five oocytes per treatment were performed. The error bars indicate the SEMs.

rGH gene is expressed only in somatotrophic cells of the anterior pituitary gland, gene regulation at rGH_3TRE, the TRE from the third intron, presumably also requires cell-specific auxiliary factors of known or unknown origin. For this reason, we sought to reconstitute the nuclear environment of anterior pituitary cells in oocytes. *Xenopus* oocytes possess the experimentally attractive feature of correctly targeting nuclear proteins, injected into the oocyte cytoplasm, to the nucleus (see, for example, Ref. 39). In this experiment, nuclear protein extract from ovine anterior pituitary cells was injected into the oocyte cytoplasm 5 h before nuclear microinjection of gene templates.

When oocytes were microinjected with nuclear protein extract, both the hormone-independent and hormone-dependent activator functions of rTRα were induced at rGH_3TRE (Fig. 5A). Surprisingly, under the same conditions, v-*Erb*A acted as a constitutive activator at rGH_3TRE, whose strength of activation exceeded that of rTRα (Fig. 5A). Increasing the amount of v-*erb*A expression template injected did not enhance this response further (data not shown). In contrast, v-*Erb*A did not affect basal promoter activity of this TRE-reporter construct in the absence of nuclear protein extract (Fig. 5A).

These findings suggested that auxiliary factor(s), present in the injected nuclear protein extract from anterior pituitary cells, significantly altered the effect of both rTRα and v-*Erb*A on reporter gene regulation at rGH_3TRE in oocytes. Two plausible candidates for this auxiliary role are the pituitary-specific transcription factor Pit-1/GHF-1 and RXR. To test this possibility, expression vectors for either Pit-1/GHF-1 or hRXRα were coinjected with rTRα or v-*erb*A constructs, and reporter gene transcription regulated by rGH_3TRE was assayed. Transcription was weakly induced by hRXRα alone, while Pit-1/GHF-1 had no significant effect on basal promoter activity (Fig. 5B). The activity patterns of rTRα and v-*Erb*A observed in the presence of nuclear protein extract of anterior pituitary cells were not replicated in the presence of hRXRα or Pit-1/GHF-1 (Fig. 5B). Instead, coexpression of hRXRα or Pit-1, together with rTRα, reduced *trans*-activation of rGH_3TRE (Fig. 5B) compared with levels of *trans*-activation by rTRα alone (Figs. 3A and 5A). Pit-1 repressed induction of rGH_3TRE by liganded rTRα below basal transcription (Fig. 5B). Compared with levels in the presence of v-*Erb*A only (Fig. 5A), coexpression of hRXRα with v-*Erb*A induced transcription slightly, while Pit-1 had no effect (Fig. 5B). In the presence of hRXRα, transcription was induced equally by rTRα or v-*Erb*A. These effects were not further investigated, since this series of experiments was carried out to test hRXRα and Pit-1 as potential candidates for conferring

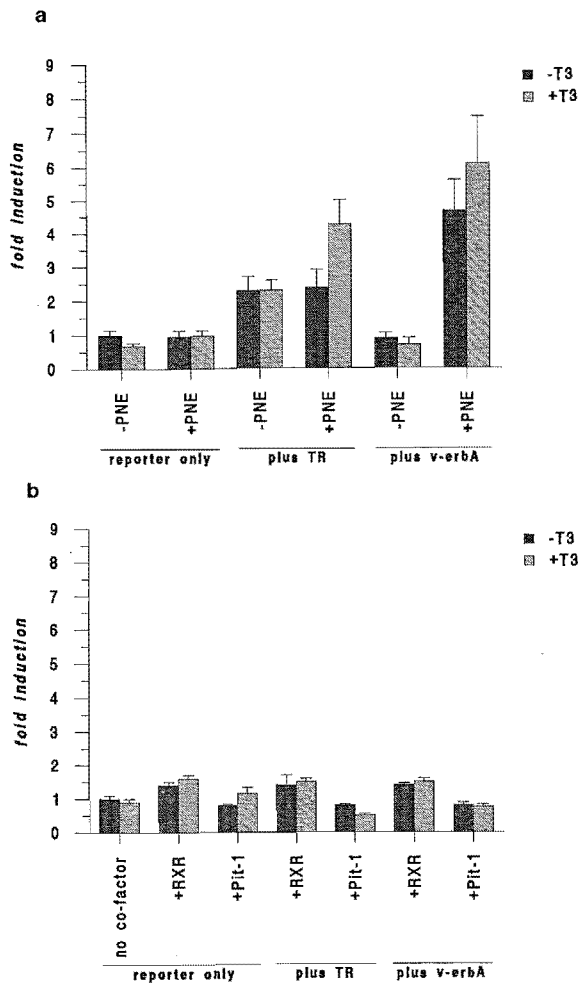

Fig. 5. Pituitary-Specific Transcriptional Regulation of rGH_3TRE by $rTR\alpha$ and v-*Erb*A

A, Action of $rTR\alpha$ and v-*Erb*A at rGH_3TRE in the presence of pituitary nuclear extract from ovine anterior pituitary cells. Oocytes were either microinjected with 20 nl nuclear protein extract into the cytoplasm 5 h before nuclear injection of gene templates (+PNE) or microinjected with the gene templates in the absence of nuclear protein extract (−PNE). Oocytes were then cultured in the absence or presence of 100 nM T_3 ($-T_3$, $+T_3$) after microinjection with 2 ng ΔMTV-rGH_3TRE-CAT reporter gene construct alone (reporter only), or together with 0.5 ng $rTR\alpha$ (plus TR) or v-*erb*A expression vector (plus v-*erb*A). After 24 h, oocytes were assayed individually for CAT protein by ELISA. Basal transcription rates of the CAT reporter construct in the absence of nuclear protein extract, exogenous TR, v-*Erb*A, and T_3 (reporter only, −PNE, $-T_3$) were arbitrarily assigned the value 1. B, Action of $rTR\alpha$ and v-*Erb*A at rGH_3TRE in the presence of $hRXR\alpha$ or Pit-1/GHF-1. Oocytes were microinjected with 2 ng ΔMTV-rGH_3TRE-CAT reporter gene construct only (reporter only, no cofactor), or together with 2.5 ng expression vector for either $hRXR\alpha$ (+RXR) or Pit-1/GHF-1 (+Pit-1). Alternatively, 0.5 ng expression plasmid for $rTR\alpha$ (plus TR) or v-*erb*A (plus v-*erb*A) was microinjected with ΔMTV-rGH_3TRE-CAT, and 2.5 ng expression vector for $hRXR\alpha$ (+RXR) or Pit-1/GHF-1 (+Pit-1) were added. Oocytes were cultured with and without T_3 ($+T_3$, $-T_3$). After 24 h, oocytes were assayed individually for CAT protein by ELISA. Basal transcription rates of the CAT reporter construct in the absence of cofactors and T_3 (reporter only, no cofactor, $-T_3$) were arbitrarily assigned the value 1. In both panels A and B, three independent experiments (five oocytes per treatment) were performed, and the mean fold induction over basal transcription was calculated. The error bars indicate the SEMs.

positive T_3-responsiveness on $rTR\alpha$, and strong constitutive activator function on v-*Erb*A, at rGH_3TRE.

Taken together, these results suggest that auxiliary factor(s), other than RXR and Pit-1/GHF-1, present in nuclear protein extracts of anterior pituitary cells, are necessary for T_3 induction of rGH_3TRE by $rTR\alpha$ and conversion of v-*Erb*A into a constitutive activator at this TRE.

DISCUSSION

Both α- and β-Isoforms of TR Act as Constitutive Activators in Oocytes

In *Xenopus* oocytes, $rTR\alpha$ acted as a ligand-independent transcriptional activator at four positively regulated TREs (lysTRE, malTRE, TREp, and rGH_3TRE). With the exception of rGH_3TRE, addition of T_3 led to a further increase in CAT reporter gene expression by $rTR\alpha$. The 3.4- to 7-fold T_3-dependent reporter gene induction observed in oocytes was comparable to activation in mammalian cells (13, 14, 40). Human $TR\beta$ also acted as a weak hormone-independent activator at TREp but was not further inducible by hormone. The observed differences in ligand-independent activation function between $rTR\alpha$ and $hTR\beta$ are most likely due to structural divergence in the N-terminal domain (5).

Both ligand-independent and ligand-induced activation functions (7–10, 41) exhibit distinct cell type and promoter context specificity in TR and other receptors belonging to the steroid/thyroid hormone receptor gene superfamily (Ref. 11; for further references, see Ref. 6). Constitutive *trans*-activation by TR has also been shown in the yeast, *S. cerevisiae* (11, 26), and in *in vitro* transcription assays using extracts from NT2 embryonal carcinoma cells or Namalwa B cells (42). Taken together with these results, the ligand-independent activation function of TR in oocytes points to a possible requirement of inhibitory auxiliary factor(s) for transcriptional repression. The finding that gene activation by unliganded TR at the clone 122 TREs was not reversed by T_3 in oocytes, while being suppressed by ligand in CV1 cells (10), also points to a requirement for an inhibitory auxiliary factor for the suppression of *trans*-activation by TR. We have shown that $hRXR\alpha$ enhances both ligand-independent and ligand-induced activation functions of $rTR\alpha$ and $hTR\beta$ at TREp, in analogy to the enhancement of both activation functions of $hTR\beta$ by $hRXR\alpha$ seen in the yeast (43). Thus, $RXR\alpha$ is not the inhibitory factor in question. Our results are in close agreement with recent evidence suggesting that an inhibitory factor sup-

presses *trans*-activation by unliganded TR in mammalian cells (19, 44) and which also showed that RXR was not the major inhibitory factor in two cell lines tested (44).

The v-*erb*A Protein Displays a Novel Pattern of Activity in Oocytes

Conversion of TR and v-*Erb*A into ligand-independent transcriptional activators has been shown to be mediated either by cell-specific protein-protein interactions (8, 11) or by direct DNA binding (9, 10). A constitutive activation function has also been identified in the N-terminal region of v-*Erb*A (9). Under conditions that induce the constitutive activation function of TR, v-*Erb*A also acts as a constitutive activator in yeast (11) and in mammalian cells (9). In contrast, we show here that the oocyte-specific cellular background induces the ligand-independent activation function of rTRα, but not of v-*Erb*A, at four positive TREs, suggesting that induction of this function can be differentially controlled in TRα and v-*Erb*A within the same cellular context. On the other hand, both rTRα and v-*Erb*A constitutively activated reporter gene transcription at the complex clone 122 TREs (Fig. 6). This TRE belongs to the same class of response elements as the TRE found in the Rous sarcoma virus (RSV)-long terminal repeat (LTR), which mediates strong T_3-independent activation by TR and v-*Erb*A in HeLa cells (9). Binding to this type of TRE induces a conformational change and renders the constitutive N-terminal activation domain accessible to the transcription machinery (9). In summary, expression of TR and v-*erb*A in oocytes induced the constitutive activation function only in TR at positive TREs, whereas this function was activated in both TR and v-*Erb*A at the clone 122 TREs

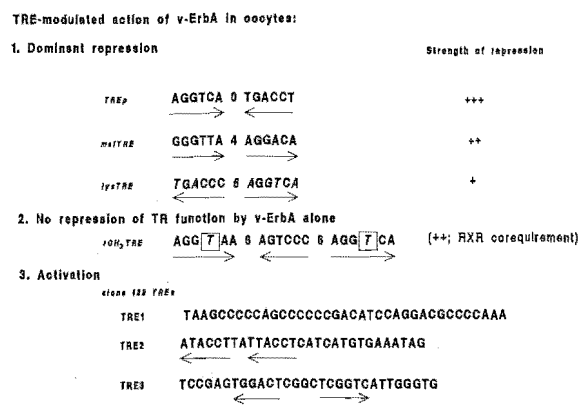

Fig. 6. Half-Site Sequences of TREs

The identity of the nucleotide residue at the fourth position of the two primary half-sites in rGH$_3$TRE is indicated (*boxed italicized letter*) (25). Intervening nucleotides between half-sites are indicated by *numbers*. *Arrows* indicate orientation of half-sites. Differential levels of dominant repression are expressed in relative terms; (+++) indicates complete repression, (++) indicates moderate repression, and (+) indicates weak repression by v-*Erb*A.

in the same cellular context. It follows that the responses of the ligand-independent activation domains of TR and v-*Erb*A to cell-specific and TRE-mediated induction are not equivalent.

In oocytes, v-*Erb*A did not repress basal promoter activity under control of all four positive TREs tested (TREp, lysTRE, malTRE, and rGH$_3$TRE). Like TR, the v-*erb*A protein possesses a repressor function located in the hinge domain (18). Transcriptional repression by TR is observed on minimal promoters containing a TATA box and TRE sequence (12, 13), and cell-free transcription assays have shown that unliganded TR functions as an active repressor of T_3-responsive genes by inhibiting the formation of a functional preinitiation complex (15). Presumably, repression by TR and v-*Erb*A occurs by a similar mechanism, namely, by interference with general transcription factors. In oocytes, protein-protein interactions between the repressor domain of v-*Erb*A and the general transcription machinery might be prevented by a lack of auxiliary inhibitory factor(s) or cell-specific characteristics of the general transcription complex.

Dominant Negative Repression by v-*Erb*A Depends on the Nature of the TRE

The action of v-*Erb*A at rGH$_3$TRE has not been tested in mammalian cells to our knowledge. In oocytes, v-*Erb*A failed to repress TR-mediated induction at rGH$_3$TRE. At the same time, v-*Erb*A dominantly repressed *trans*-activation by TR at three other positive TREs with diverse half-site characteristics (malTRE, lysTRE, and TREp). The TRE-modulated action of v-*Erb*A in oocytes is summarized in Fig. 6. Although both primary half-sites of rGH$_3$TRE possess a favorable thymidine nucleotide at the fourth position (25, 31), and the sequence of the major binding site is identical to the AGGTC/GA consensus motif (Fig. 6), it appears that v-*Erb*A does not successfully compete with rTRα for binding due to the structure of rGH$_3$TRE. As shown by other workers, heterodimerization with RXR-related factors can be essential for the dominant negative action of v-*Erb*A (40, 45). In the present study, a reduced level of *trans*-activation of rGH$_3$TRE by rTRα and v-*Erb*A was observed in the presence of hRXRα. However, the significance of this finding is presently unclear, since hRXRα also reduced *trans*-activation by rTRα alone at this TRE.

An Activity in Nuclear Protein Extracts from Anterior Pituitary Cells Conferred T_3-Responsiveness to rTRα and Converted v-*Erb*A into a Constitutive Activator at rGH$_3$TRE

Our findings suggest that T_3 induction of rGH$_3$TRE is regulated in a cell-specific manner. In the presence of nuclear protein extract from anterior pituitary cells, the T_3-dependent activation function of rTRα was induced at rGH$_3$TRE in addition to T_3-independent *trans*-activation by rTRα without extract. We showed that T_3 responsiveness of rTRα was not mediated by hRXRα

or the pituitary-specific transcription factor Pit-1/GHF-1, which is required for efficient T_3-induction of the GH gene in somatotrophs (38). General mammalian-type transcription factors might have been involved in conferring T_3 responsiveness to rTRα, since rGH_3TRE is also T_3-inducible in CV1 cells (31). The tripartite rGH_3TRE, although located in the third intron of the rGH gene, exhibits a markedly higher affinity for TR, as well as greater gene induction ability, than the TRE located in the promoter region of the gene, indicating an important *in vivo* role for this TRE sequence (31). Further indications of the importance of regulatory regions in introns are the presence of a glucocorticoid-responsive element in the first intron of the human GH gene (46) and the requirement for the presence of introns in the rat GH gene for efficient expression in transgenic animals (47).

Interestingly, v-*Erb*A acted as an efficient constitutive activator at rGH_3TRE in the presence of nuclear protein extract from anterior pituitary cells, whereas it had no effect on basal promoter activity under the control of rGH_3TRE without extract. Coexpression of Pit-1/GHF-1 or hRXRα did not replicate the mode of action of the v-*erb*A protein as a constitutive activator seen with the nuclear protein extract. This finding suggests that indirect or direct interactions with an as yet unidentified nuclear factor present in pituitary cells result in a conformational change in v-*Erb*A at rGH_3TRE that induces its constitutive activation function. The conversion of v-*Erb*A into a constitutive activator by nuclear protein extract from anterior pituitary cells at rGH_3TRE suggests that the dominant negative phenotype of the v-*erb*A oncogene can be abolished by either direct or indirect interactions with other nuclear factors.

Our experimental approach illustrates how studies of exogenous receptors in oocytes, in conjunction with microinjected auxiliary proteins, may contribute to the characterization of cell-specific and TRE-mediated mechanisms that modulate the action of receptor domains.

MATERIALS AND METHODS

Plasmids

RS-rTRα, RS-v-erbA, and *tk*-TREp-CAT were gifts from R. Evans (Salk Institute for Biological Studies, La Jolla, CA). The expression vectors contain rTRα cDNA or the *gag*-v-*erb*A oncogene under the transcriptional control of the RSV-LTR (14, 48). *tk*-TREp-CAT contains a synthetic palindromic TRE linked to a herpes simplex virus *tk*-CAT fusion gene (14). The T_3-responsive CAT reporter constructs contain malTRE, lysTRE, rGH_3TRE, or TREp linked to a MTV (ΔMTV) LTR-CAT fusion gene (ΔMTV-CAT)(24). The pTPT-CAT reporter construct was a gift from R. N. Eisenman (Fred Hutchinson Cancer Research Center, Seattle, WA) and contains a 450-base pair *Sau*3A-*Bam*HI fragment from clone 122, spanning all three TREs, cloned into pBLCAT3 (10). pRSV-Pit-1 was a gift from H. H. Samuels (New York University Medical Center, New York, NY) and contains a cDNA clone of the rat Pit-1/GHF-1 gene linked to the RSV LTR (49). The RSh-TRβ construct contains the wild type hTRβ gene, and the RSh-TRβ C122 > A expression plasmid contains an *in vitro*-generated mutant hTRβ gene (alanine instead of cysteine at position 122), both under control of the RSV LTR (24). The RSh-RXRα expression vector contains hRXRα cDNA linked to the RSV LTR. ΔMTV-CAT contains a CAT reporter gene driven by the ΔMTV LTR promoter (48). pRSV-*lacZ* was obtained from M. Harkey (University of Washington, Seattle, WA) and contains the *Escherichia coli* β-galactosidase gene under control of the RSV LTR (50).

Microinjections and ELISAs

A lobe of ovary was surgically removed from an adult female *Xenopus laevis* and processed as described (51). Microinjections were performed according to published methods with modifications (51, 52). Stage V oocytes were microinjected with 20 nl plasmid DNA in TE (10 mM Tris-HCl, 1 mM EDTA), pH 8.0, into the nucleus by the 'blind' injection method with the needle inserted in the center of the animal pole (27, 53). After microinjection, oocytes were incubated in OR-2 (51) medium for 20–24 h at 18 C in the presence or absence of 100 nM T_3 (Sigma Chemical Co., St Louis, MO). Healthy oocytes (uniform pigment, not mottled) were homogenized singly in 200 μl 0.25 M Tris buffer, pH 7.5, and centrifuged at 10,000 × *g* in a microfuge at 4 C for 10 min. The supernatant was then centrifuged as before. This extract was used in the determination of CAT and β-galactosidase protein expression levels by ELISA according to the manufacturer's specifications (5 Prime-3 Prime, Inc., Boulder, CO). The concentration of total protein in extracts from single oocytes was adjusted to a concentration of 175–200 μg/ml by 1:1 dilution with dilution buffer. Microwells coated with rabbit polyclonal antibody specific to CAT protein or specific to the *E. coli* β-galactosidase protein (β-Gal) were incubated with extracts from microinjected oocytes, uninjected controls, and appropriate pure CAT or β-Gal protein standards. Biotinylated secondary antibody to CAT or β-Gal was then bound to the primary antibody-antigen complex. After washing, bound biotinylated antibody was quantitated colorimetrically by incubation with streptavidin-conjugated alkaline phosphatase and p-nitrophenyl phosphate as substrate. The wells were read at 405 nm against the reagent blank in a microtiter well reader (model EL 311, Bio-Tek Instruments, Winooski, VT). For each assay, a standard curve utilizing four pure protein standards was prepared, to ensure that CAT or β-Gal concentrations of sample extracts fell within the linear range of the assay. All experiments were repeated two to three times with different animals since transcriptional activity may vary between different batches of oocytes.

ELISA for Total T_3

Ten oocytes per sample, either incubated in 100 nM T_3 for 20 h and subsequently washed three times in OR-2 or cultured in OR-2 only, were homogenized in 100 μl 0.25 M Tris buffer, pH 7.5, and centrifuged at 10,000 × *g* in a microfuge at 4 C for 10 min. The supernatant was then centrifuged as before. This extract was used in the quantitative determination of total T_3, both bound and free hormone, in oocytes by ELISA (Enzymun-Test T_3, Boehringer Mannheim, Auckland, New Zealand). Test tubes coated with streptavidin were incubated with oocyte extracts or T_3 standards, together with anti-T_3 antibody-horseradish peroxidase conjugate. T_3 standards were prepared in 0.25 M Tris buffer, pH 7.5, at 0, 0.1, 1, 10, and 100 nM. Binding of sample T_3 to anti-T_3 antibodies was then competed by addition of biotinylated T_3 polyhapten, which anchors previously unbound anti-T_3 antibodies to the streptavidin-coated test tube wall. After washing, bound anti-T_3 antibody-horseradish peroxidase conjugate was quantitated colorimetrically by incubation with 2,2′-azino-di-[3-ethyl-benzthiazoline sulfonate (6)]/H_2O_2. The samples were

transferred to microcuvettes and read against buffer at 420 nm in a spectrophotometer. For each assay, a standard curve utilizing the five T_3 standards was prepared. Two independent determinations were carried out with oocytes from two different animals, and four replicates (10 oocytes per replicate) were assayed in each determination.

Preparation of Nuclear Protein Extracts from Ovine Anterior Pituitary Cells and Cytoplasmic Injection

Nuclear protein extracts were prepared essentially as described by Maxon *et al.* (39). All procedures were carried out at 4 C. Ten milliliters of sheep anterior pituitary cells maintained in primary cell culture (4.8×10^6 viable cells/ml) were concentrated by centrifugation, washed in PBS, and then washed once in 1.5 M glucose. The resultant partially lysed cells were homogenized gently in 10 vol low-salt buffer (4 mM magnesium acetate, 50 mM Tris, pH 7.4, 0.5% Nonidet P-40, 10 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride [PMSF]). Two to five strokes in a Dounce glass homogenizer were sufficient to complete lysis. The homogenate was overlaid (15 ml per tube) on step sucrose gradients consisting of 5 ml 1.7 M sucrose on 5 ml 2.2 M sucrose in 30-ml Nalgene tubes. The gradients were centrifuged at $5,000 \times g$ for 15 min, and the gray nuclei were collected from the interface between the two sucrose solutions. The nuclei were diluted 2-fold with a buffer containing 75 mM NaCl, 25 mM EDTA, 5 mM Tris, pH 7.5, 20 mM 2-mercaptoethanol, and 0.1 mM PMSF. Partially lysed nuclei and chromatin were collected by centrifugation at $10,000 \times g$ for 30 min. The chromatin-nuclei pellet was suspended in extraction buffer [0.45 M NaCl, 20 mM Tris, pH 7.0, 1 mM EDTA, 1 mM EGTA, 0.5 mM dithiothreitol (DTT), 0.1 mM PMSF] and vortexed for several minutes, and the insoluble material was collected by centrifugation at $10,000 \times g$ for 30 min. The supernatant was set aside, and the extraction and centrifugation were repeated on the residual nuclear material with extraction buffer containing 1 M NaCl and then 2 M NaCl. The extracts of all three salt washes were pooled and concentrated by precipitation with ammonium sulfate (0.35 g/ml), dialyzed for 24 h against several changes of injection buffer (100 mM NaCl, 20 mM Tris, pH 7.0, 5 mM $MgCl_2$, 1 mM EDTA, 0.1 mM EGTA, 5% glycerol, 0.5 mM DTT, 0.1 mM PMSF), and concentrated severalfold by centrifugation in centricon 10 microconcentrators (Amicon, Beverly, MA). Extracts were stored frozen at −80 C. Five hours before microinjection of gene templates, oocytes were microinjected with 20 nl extract into the cytoplasm.

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Address requests for reprints to: Dr. Lizabeth A. Allison, Department of Zoology, University of Canterbury, Private Bag 4800, Christchurch, New Zealand.

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REFERENCES

1. Beato M 1989 Gene regulation by steroid hormones. Cell 56:335–344
2. Sap J, Munoz A, Damm K, Goldberg Y, Ghysdael J, Leutz A, Berg H, Vennstrom B 1986 The c-erb-A protein is a high affinity receptor for thyroid hormone. Nature 324: 635–640
3. Weinberger C, Thompson CC, Ong ES, Lebo R, Grvol DJ, Evans RM 1986 The c-erbA gene encodes a thyroid hormone receptor. Nature 324:641–646
4. Chatterjee VKK, Tata JR 1992 Thyroid hormone receptors and their role in development. Cancer Surv 14:147–167
5. Glass CK, Rosenfeld MG 1991 Regulation of gene transcription by thyroid hormones and retinoic acid. In: Foulkes G, Cohen P (eds) The Hormonal Control of Gene Transcription. Elsevier, Amsterdam, vol 6:299–327
6. Gronemeyer H 1992 Control of transcription activation by steroid hormone receptors. FASEB J 6:2524–2529
7. Forman BM, Samuels HH 1990 Interactions among a subfamily of nuclear hormone receptors: the regulatory zipper model. Mol Endocrinol 4:1293–1301
8. Forman BM, Yang C-R, Stanley F, Casanova J, Samuels HH 1988 c-erbA protooncogenes mediate thyroid hormone-dependent and independent regulation of the rat growth hormone and prolactin genes. Mol Endocrinol 2:902–911
9. Saatcioglu F, Deng T, Karin M 1993 A novel cis element mediating ligand-independent activation by c-ErbA: Implications for hormonal regulation. Cell 75:1095–1105
10. Bigler J, Eisenman RN 1994 Isolation of a thyroid hormone-responsive gene by immunoprecipitation of thyroid hormone receptor-DNA complexes. Mol Cell Biol 14:7621–7632
11. Privalsky ML, Sharif M, Yamamoto KR 1990 The viral erbA oncogene protein, a constitutive repressor in animal cells, is a hormone-regulated activator in yeast. Cell 63: 1277–1286
12. Baniahmad A, Kohne AC, Renkawitz R 1992 A transferable silencing domain is present in the thyroid hormone receptor, in the v-erbA oncogene product and in the retinoic acid receptor. EMBO J 11:1015–1023
13. Baniahmad A, Steiner C, Kohne AK, Renkawitz R 1990 Modular structure of a chicken lysozyme silencer: involvement of an unusual thyroid hormone receptor binding site. Cell 61:505–514
14. Damm K, Thompson CC, Evans RM 1989 Protein encoded by v-erbA functions as a thyroid hormone receptor antagonist. Nature 339:593–597
15. Fondell JD, Roy AL, Roeder RG 1993 Unliganded thyroid hormone receptor inhibits formation of a functional preinitiation complex: implications for active repression. Genes Dev 7:1400–1410
16. Sap J, Munoz A, Schmitt J, Stunnenberg H, Vennstrom B 1989 Repression of transcription mediated by a thyroid hormone response element by the v-erb-A oncogene product. Nature 340:242–244
17. Zenke M, Munoz A, Sap J, Vennstrom B, Beug H 1990 v-erbA oncogene activation entails the loss of hormone-dependent regulator activity of c-erbA. Cell 61:1035–1049
18. Damm K, Evans RM 1993 Identification of a domain required for oncogenic activity and transcriptional suppression by v-erbA and thyroid hormone receptor α. Proc Natl Acad Sci USA 90:10668–10672
19. Baniahmad A, Leng X, Burris TP, Tsai SY, Tsai M-J, O'Malley BW 1995 The τ4 activation domain of the thyroid hormone receptor is required for release of a putative corepressor(s) necessary for transcriptional silencing. Mol Cell Biol 15:76–86
20. Lazar MA, Berrodin TJ, Harding HP 1991 Differential DNA binding by monomeric, homomeric, and potentially het-

eromeric forms of the thyroid hormone receptor. Mol Cell Biol 11:5005–5015
21. Mangelsdorf DJ, Evans RM 1992 Retinoid receptors as transcription factors. In: McKnight SL and Yamamoto KR (ed) Transcriptional regulation. Monograph 22, Cold Spring Harbor Laboratory Press, Cold Spring Harboe, NY, pp. 1137–1167
22. Privalsky ML 1992 v-*erb* A, nuclear hormone receptors, and oncogenesis. Biochim Biophys Acta 1114:51–62
23. Sharif M, Privalsky ML 1991 v-*erb* A oncogene function in neoplasia correlates with its ability to repress retinoic acid receptor action. Cell 66:885–893
24. Nelson CC, Faris JS, Hendy SC, Romaniuk PJ 1993 Functional analysis of the amino acids in the DNA recognition α-helix of the human thyroid hormone receptor. Mol Endocrinol 7:1185–1195
25. Chen H-W, Smit-McBride Z, Lewis S, Sharif M, Privalsky ML 1993 Nuclear hormone receptors involved in neoplasia: ErbA exhibits a novel DNA sequence specificity determined by amino acids outside of the zinc finger region. Mol Cell Biol 13:2366–2376
26. Smit-McBride Z, Privalsky ML 1993 Functional domains of the v-*erb*A protein necessary for oncogenesis are required for transcriptional activation in *Saccharomyces cerevisiae*. Oncogene 8:1465–1475
27. Gurdon JB, Melton DA 1981 Gene transfer in amphibian eggs and oocytes. Annu Rev Genet 15:189–218
28. Kawahara A, Baker BS, Tata JR 1991 Developmental and regional expression of thyroid hormone receptor genes during *Xenopus* development. Development 112:933–943
29. Banker DE, Bigler J, Eisenman RN 1991 The thyroid hormone receptor gene (c-*erb*Aα) is expressed in advance of thyroid gland maturation during early embryonic development of *Xenopus* laevis. Mol Cell Biol 11:5079–5089
30. Petty KJ, Desvergne B, Mitsuhashi T, Nikodem VM 1990 Identification of a thyroid hormone response element in the malic enzyme gene. J Biol Chem 265:7395–7400
31. Sap J, de Magistris L, Stunnenberg H, Vennstrom B 1990 A major thyroid hormone response element in the third intron of the rat growth hormone gene. EMBO J 9:887–896
32. Eliceiri BP, Brown DD 1994 Quantitation of endogenous thyroid hormone receptors α and β during embryogenesis and metamorphosis in *Xenopus laevis*. J Biol Chem 269:24459–24465
33. Holloway JM, Glass CK, Adler S, Nelson CA, Rosenfeld MG 1990 The C'-terminal interaction domain of the thyroid hormone receptor confers the ability of the DNA site to dictate positive or negative transcriptional activity. Proc Natl Acad Sci USA 87:8160–8164
34. Fuerstenberg S, Leitner I, Schroeder C, Schwarz H, Vennström B, Beug H 1992 Transcriptional repression of band 3 and CAII in v-*erb*A transformed erythroblasts accounts for an important part of the leukaemic phenotype. EMBO J 11:3355–3365
35. Ptashne M 1988 How eukaryotic transcriptional activators work. Nature 335:683–689
36. Forrest D, Munoz A, Raynoschek C, Vennstrom B, Beug H 1990 Requirement for the C-terminal domain of the v-*erb*A oncogene protein for biological function and transcriptional repression. Oncogene 5:309–316
37. Privalsky ML, Boucher P, Koning A, Judelson C 1988 Genetic dissection of functional domains within the avian erythroblastosis virus v-*erb* A oncogene. Mol Cell Biol 8:4510–4517
38. Lefevre C, Imagawa M, Dana S, Grindlay J, Bodner M, Karin M 1987 Tissue-specific expression of the human growth hormone gene is conferred in part by the binding of a specific *trans*-acting factor. EMBO J 6:971–981
39. Maxon R, Ito M, Balcells S, Thayer M, French M, Lee F, Etkin L 1988 Differential stimulation of sea urchin early and late H2B histone gene expression by a gastrula nuclear extract after injection into *Xenopus laevis* oocytes. Mol Cell Biol 8:1236–1246
40. Hermann T, Hoffman B, Piedrafita FJ, Zhang X-H, Pfahl M 1993 v-*erb*A requires auxiliary protein for dominant negative activity. Oncogene 8:55–65
41. Barettino D, Ruiz MV, Stunnenberg HG 1994 Characterization of the ligand-dependent transactivation domain of thyroid hormone receptor. EMBO J 13:3039–3049
42. Lee IJ, Driggers PH, Medin JA, Nikodem VM, Ozato K 1994 Recombinant thyroid hormone receptor and retinoid X receptor stimulate ligand-dependent transcription *in vitro*. Proc Natl Acad Sci USA 91:1647–1651
43. Lee JW, Moore DD, Heyman RA 1994 A chimeric thyroid hormone receptor constitutively bound to DNA requires retinoid X receptor for hormone-dependent transcriptional activation in yeast. Mol Endocrinol 8:1245–1252
44. Casanova J, Helmer E, Selmi-Ruby S, Qi J-S, Au-Fliegner M, Desai-Yajnik V, Koudinova N, Yarm F, Raaka BM, Samuels HH 1994 Functional evidence for ligand-dependent dissociation of thyroid hormone and retinoic acid receptors from an inhibitory cellular factor. Mol Cell Biol 14:5756–5765
45. Chen H-W, Privalsky ML 1993 The *erb*A oncogene represses the actions of both retinoid X and retinoid A receptors but does so by distinct mechanisms. Mol Cell Biol 13:5970–5980
46. Slater EP, Rabenau O, Harin M, Baxter JD, Beato M 1985 Glucocorticoid receptor binding and activation of a heterologous promoter by dexamethasone by the first intron of the human growth hormone gene. Mol Cell Biol 5:2984–2992
47. Brinster RL, Allen JM, Behringer RR, Gelinas RE, Palmiter RD 1988 Introns increase transcriptional efficiency in transgenic mice. Proc Natl Acad Sci USA 85:836–840
48. Thompson CC, Evans RM 1989 Trans-activation by thyroid hormone receptors: Functional parallels with steroid hormone receptors. Proc Natl Acad Sci USA 86:3494–3498
49. Fox SR, Jong MTC, Casanova J, Ye Z-S, Stanley F, Samuels HH 1990 The homeodomain protein, Pit-1/GHF-1, is capable of binding to and activating cell-specific elements of both the growth hormone and prolactin gene promoters. Mol Endocrinol 4:1069–1080
50. Gorman C, Padmanabhan R, Howard BH 1983 High efficiency DNA-mediated transformation of primate cells. Science 221:551–553
51. Allison LA, Romaniuk PJ, Bakken AH 1991 RNA-protein interactions of stored 5S RNA with TFIIIA and ribosomal protein L5 during *Xenopus* oogenesis. Dev Biol 144:129–144
52. Allison LA, North MT, Murdoch KJ, Romaniuk PJ, Deschamps S, le Maire M 1993 Structural requirements of 5S rRNA for nuclear transport, 7S ribonucleoprotein particle assembly, and 60S ribosomal subunit assembly in *Xenopus* oocytes. Mol Cell Biol 13:6819–6831
53. Colman A 1984 Expression of exogenous DNA in *Xenopus* oocytes. In: Hames BD, Higgins SJ (eds) Transcription and Translation. IRL Press, Oxford, Washington DC, pp 49–69

Appendix 2

Solutions

O-R2, pH 7.6 (working concentrations):

82.5 mM NaCl
2.5 mM KCl
1.0 mM $CaCl_2$
1.0 mM $MgCl_2$
1.0 mM Na_2HPO_4
5.0 mM Hepes
3.8 mM NaOH

O-R2 working solution:

10 ml Stock A
10 ml Stock B
80 ml sterile dd H_2O

O-R2 Stock A (10X):

825 mM NaCl	4.820 g
25 mM KCl	0.186 g
10 mM Na_2HPO_4	0.142 g
50 mM Hepes	1.190 g
38 mM NaOH	3.8 ml (1M stock)*

* freshly made, 0.4 g/10 ml ddH_2O

O-R2 Stock B (10X):

10 mM $MgCl_2$	0.203 g (or 10 ml of 100 mM)
10 mM $CaCl_2$	0.147 g (or 10 ml of 100 mM)

NET-2:

50 mM Tris-HCl, pH 7.4
150 mM NaCl
0.05% NP-40 (add after autoclaving)